The role of efflux and influx transporters in the disposition of digitalis-like compounds

Elnaz Gozalpour

About the cover:

Digitalis-like compounds, extracted from plants or animals, were used as arrow poisons by African hunters. Proteins located in cellular plasma membrane are responsible for transport of these compounds to prevent their toxicity. Seeing yellow halos (a vision disturbance called xanthopsia) is one of digitalis toxicity symptoms in digoxin-prescribed patients. On the cover, the arrow, poisoned by purple foxglove, lily of the valley and *bufo bufo* toad targeting drug transporter proteins, represents the aim of this thesis. The yellow halos at the cover background represent part of Vincent van Gogh's painting called "Starry Night over the Rhone". There are some stories explaining the plenty of yellow halos in van Gogh's paintings were the result of digitalis-like compounds prescription for his epilepsy.

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The role of efflux and influx transporters in the disposition of digitalis-like compounds

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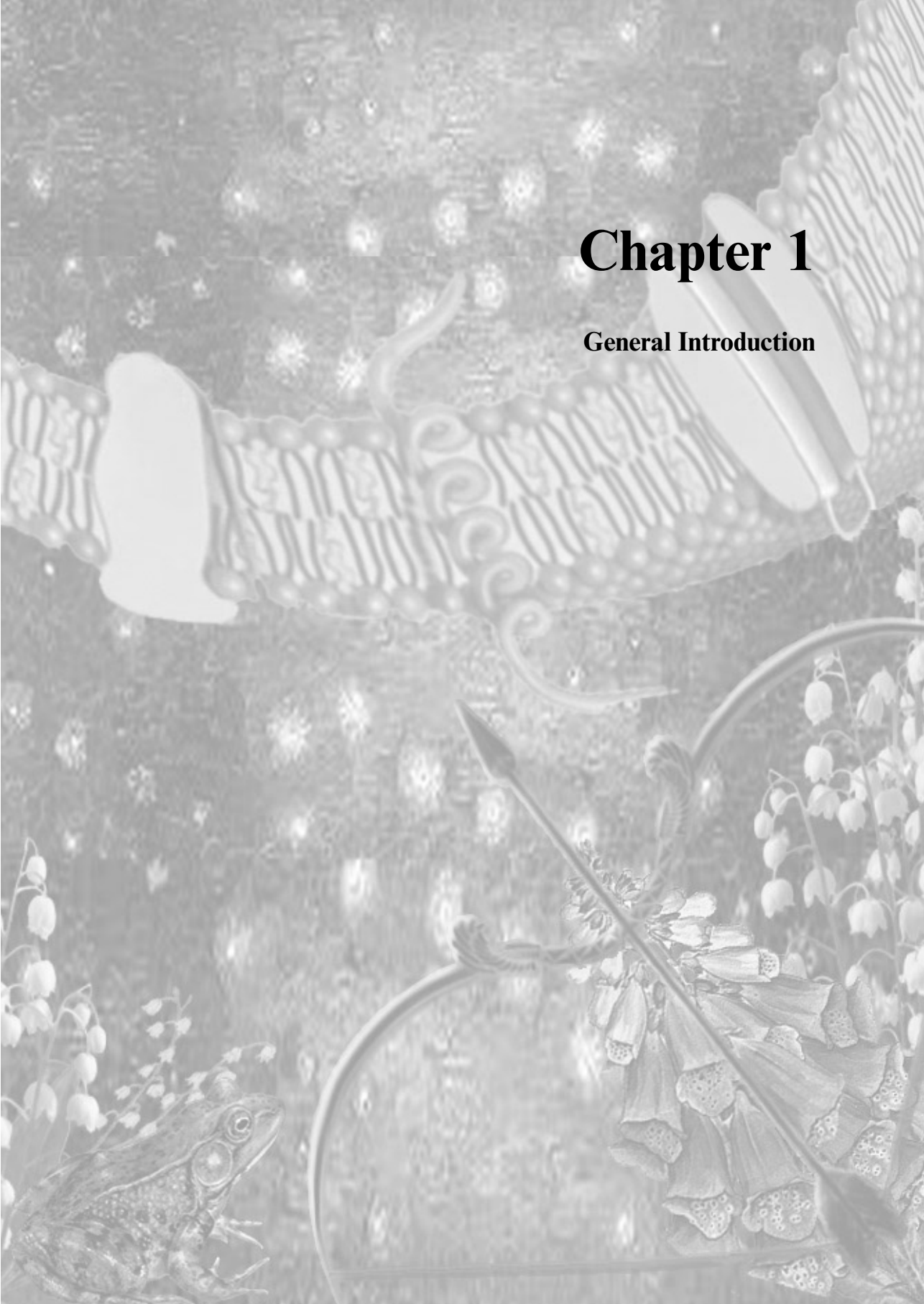

Chapter 1

General Introduction

Historical perspective

The curative application of digitalis-like compounds (DLCs) dates back to 1550 BC when squill plant was known as diuretic, heart tonic and rat poison (Luderitz, 2005). Plant extracts containing DLCs were also recommended for headache, spasm, epilepsy, and skin diseases in the 13th century (Somberg *et al.*, 1986; Silverman, 1989). In Chinese medicine, the skin powder of the *Bufo gargarizans* toad was used as a medication that apparently contained bufalin (Somberg *et al.*, 1986). Furthermore, the plant and toad-derived DLCs were used as an arrow poison in African tribes and foxglove extracts were used as a strong poison by ancient Romans (Cassels, 1985).

In the 18th century the British medical doctor and botanist, William Withering, discovered that digitalis was the prominent part of an herbal remedy that was introduced to him by a rural woman called "Mother Hutton" in Shropshire (England) for treatment of edema. In December 1775, a fifty year old man, who was suffering from accumulation of fluid and difficulties with breathing (called dropsy in that time), was the first patient prescribed digitalis by William Withering. Since then, Withering began his study and experiments on foxglove extract containing digitalis and published the book "An account of the Foxglove, and some of its medical uses: with practical remarks on dropsy, and other diseases" in 1785 (Rahimtoola, 1975; Krikler, 1985; Rossner, 2006).

After treatment of 163 documented patients, Withering described medication preparation from purple foxglove (*Digitalis purpurea*), different sensitivity of patients to the medication, dose-response characteristics and some adverse effects of foxglove extract such as vomiting, observation of yellow and green objects, increased urine secretion, slow heart rhythm and death (Fisch & Knoebel, 1985; Breckenridge, 2006). Although he considered this medication as a diuretic agent at first, he also mentioned the effect on cardiac pulses (Fulton, 1953; Kinne-Saffran & Kinne, 2002). In addition, he combined the treatment with opium to diminish vomiting and nausea effects of the drug (Bessen, 1986; Somberg *et al.*, 1986).

After Withering's death in 1799, disregarding his warnings about digitalis toxicity and dosing, digitalis therapy was extended all over Britain to treat all forms of edema and neurological disorders, which caused the death of many patients (Somberg *et al.*, 1986). Due to its toxicity, digitalis therapy was less accepted in France and Germany during the 19th century. In the US, purple foxglove was grown by Hall Jackson who received seeds of the plant from Withering after studying his book and it was used for therapeutic purposes. The first extracted DLC was strophanthinic acid isolated by Richard Fraser in 1885 from Strophanthus plant, which was used as arrow poison by some African tribes. The production of injectable strophanthinic acid by Albert Fraenkel opened the way to the therapeutic use of DLCs in cardiology (Luderitz, 2005).

Structure

DLCs consist of two moieties: a genin or aglycone moiety and a sugar moiety. The genin core is composed of steroid rings (A-D rings) with a *cis* conformation between A/B and C/D rings and a *trans* combination of B/C (Figure 1). However, in some plants (*Asclepiadaceae*) the A/B ring has a *trans* combination (e.g. uscharin and calactin) (Thomas *et al.*, 1974; Mijatovic *et al.*, 2007). The genin moiety has methyl groups at position C10 and C13 and 2-6 hydroxyl groups at different positions. The aglycone moiety of DLCs can bind to 1-4 sugar molecules such as digitoxose, rhamnose and mannose to form glycosides (Hussain *et al.*, 2006). Moreover, the lactone ring at position 17β discriminates cardenolides with a butyrolactone ring (5-membered unsaturated γ-butyrolactone) from bufadienolides with a pyrone ring (6-membered double unsaturated δ-valerolactone) (Figure 1). Cardenolides such as digoxin, digitoxin, and oleandrin are derived from foxglove and Oleander, whereas bufadienolides such as bufalin originate from toads (Vaklavas *et al.*, 2011) (Table 1). The aglycone core of the cardiac glycoside conserves the cardiotonic activity and the sugar moiety plays a role in its pharmacokinetic properties and water solubility (Thomas *et al.*, 1974; Smith *et al.*, 1984).

Interaction with Na,K-ATPase

DLCs inhibit Na,K-ATPase, which was first characterized by Schatzmann in 1965 (Schatzmann, 1953; Schatzmann & Rass, 1965). Na,K-ATPase, also known as sodium pump, is an antiporter enzyme in the plasma membrane, which regulates the resting potential and volume of the cells. It pumps three Na^+ ions out of the cell and takes up two K^+ ions by direct hydrolysis of ATP to drive transport. Na,K-ATPase consists of two subunits: the α-subunit is the catalytic subunit of the enzyme, whereas the β-subunit plays a role in post-translational maturation and plasma membrane localization (Shull *et al.*, 1985; Sverdlov *et al.*, 1987; Katz *et al.*, 2010). The α- and β-subunits have several different isoforms (α1-4 and β1-3), which are distributed tissue-specifically. The α1 and β1 subunits are ubiquitously expressed, whereas α2 and β2 are present in muscle (skeletal, smooth and heart) and α3 is expressed in nervous tissue (Lavoie *et al.*, 1997; Katz *et al.*, 2010). Moreover, α4 is specifically expressed in testis (Blaustein *et al.*, 2009; Katz *et al.*, 2010) and β3 is present in testis, retina, liver, and lung (Malik *et al.*, 1996; Arystarkhova & Sweadner, 1997).

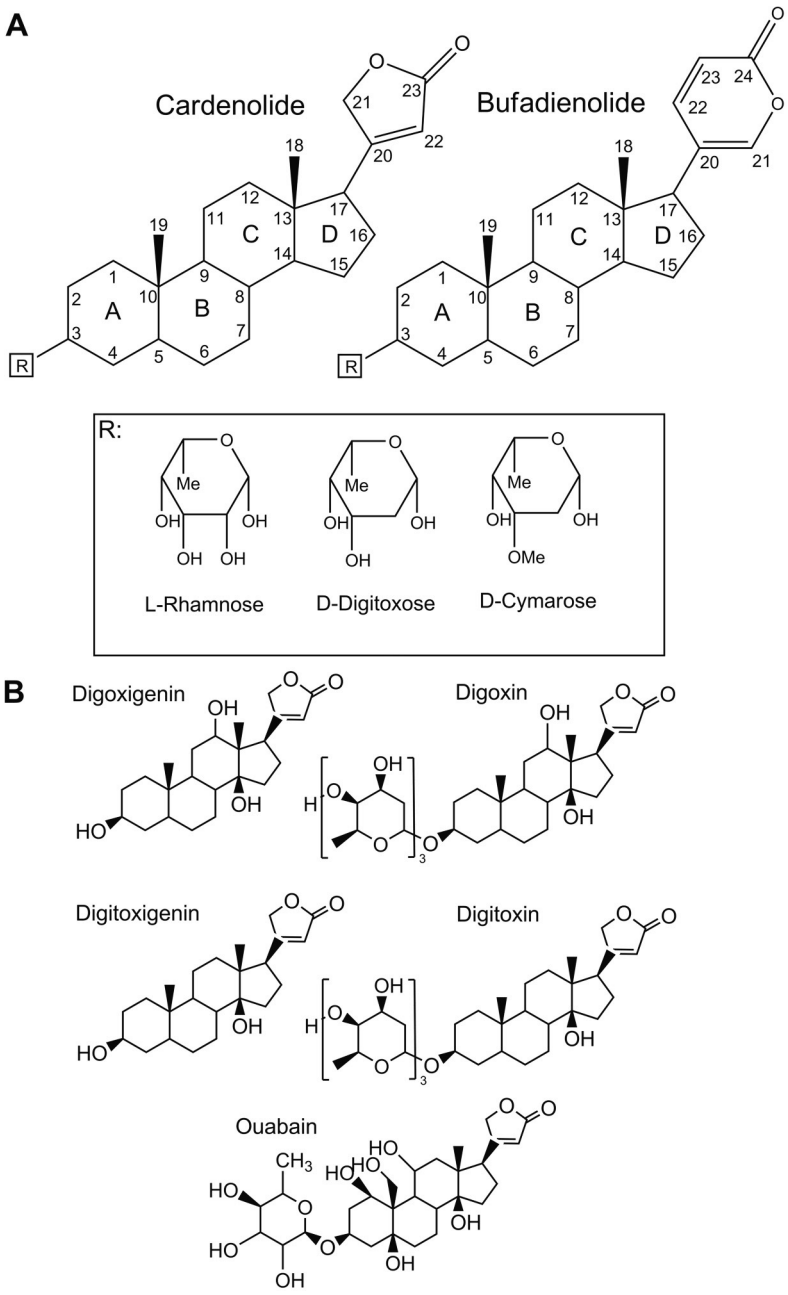

Figure 1. Chemical structure of digitalis-like compounds (DLCs). (A) Steroid core structure of DLCs illustrating four rings (A, B, C and D), carbon numbers, γ-butyrolactone and δ-valerolactone at position C17 of cardenolides and bufadienolides, respectively and a sugar moiety (R) at C3. (B) Five DLCs, digoxigenin (aglycone), digoxin, digitoxigenin (aglycone), digitoxin, and ouabain (glycoside) have been presented.

Digitalis-like compounds (DLCs) bind to the α subunit of the enzyme, and various studies have been performed to determine the selectivity for Na,K-ATPase isoforms (Lingrel & Kuntzweiler, 1994; Malik *et al.*, 1996; Kaplan, 2002). In rat, the α1 isoform is the most resistant one to ouabain (affinity of ouabain: α3 > α2 > α1) (O'Brien *et al.*, 1994; McDonough *et al.*, 2002). However, all nine combinations of human α-subunits with β-subunits have a similar high affinity for ouabain (Crambert *et al.*, 2000; Wang *et al.*, 2001; McDonough *et al.*, 2002). Comparing the structural moieties of DLCs and their inhibitory potency, different DLCs have different affinities for α subunits and it seems that the sugar moieties play a role in the pharmacodynamic profile of DLCs (Hauck *et al.*, 2009; Katz *et al.*, 2010). Recently, Weigand *et al.* showed that amino acids Met119 and Ser124, located in the first extracellular loop of the Na,K-ATPase, are responsible for the low affinity of dihydro-DLCs (DLCs with a hydrogenated lactone ring) for α2 isoform (Weigand *et al.*, 2014). There are five structural criteria for interaction of DLCs with Na,K-ATPase: (1) a *cis* conformation of the C/D rings, (2) an unsaturated lactone ring, (3) a β attachment of the lactone ring to C17, (4) a β attachment of the hydroxyl group at positions C3 and C14, and (5) a β attachment of C3 with the sugar moiety (Chen & Henderson, 1954; Brown *et al.*, 1962; Thomas *et al.*, 1974; Sevillano *et al.*, 2002).

Table 1. Number of DLCs derived from different plant or animal species

Digitalis-like compounds	Source species	Reference
Cardenolides		
Convallatoxin	*Convallaria majalis* (Lily of the valley)	(Choi *et al.*, 2006)
Cymarin	*Apocynum cannabinum* (Dogbane)	(Kupchan *et al.*, 1964)
Digitoxigenin, Digitoxin, Digoxin Digoxigenin, Gitoxigenin	*Digitalis purpurea, Digitalis lanata* (foxglove)	(Mijatovic *et al.*, 2007)
Ouabain, Ouabagenin	*Strophanthus gratus*	(Mijatovic *et al.*, 2007)
Strophanthidin, Strophanthidol	*Strophanthus Kombe*	(Grosa *et al.*, 2005)
Peruvoside	*Thevetia peruvians* (Yellow Oleander)	(Arora *et al.*, 1967)
Bufadienolides		
Bufalin	*Bufo vulgaris*	(Brubacher *et al.*, 1999)
Proscillaridin A	*Drimia maritima* (Squill)	(Gould *et al.*, 1971)

Na,K-ATPase exists in two main conformational states: E1 and E2 and ouabain has the highest affinity for the phosphorylated form of E2 (Matsui & Schwartz, 1968). It has been shown that ouabain binds to the extracellular side of Na,K-ATPase. Based on site-directed mutagenesis studies, the important amino acids for binding of ouabain to Na,K-ATPase have been identified. Two polar residues, Gln111 and Asn122 located at the extracellular border of transmembrane segments (M1 and M2) are responsible

for the sensitivity of this pump to ouabain in non-rodents (Price *et al.*, 1989). As H,K-ATPase is a closely-related enzyme to Na,K-ATPase, a chimera of these two enzymes was used to determine the ouabain-binding residues of Na,K-ATPase in a number of studies. In conclusion, Glu312, Val314, Ile315, and Gly319 located in M4, Pro778 and Phe783 located in M5 and Leu795, The797, Cys802, and Asp804 located in M6 have been reported as key amino acids of Na,K-ATPase leading to high affinity ouabain-binding chimera (Koenderink *et al.*, 2000; Qiu *et al.*, 2003; Qiu *et al.*, 2005; Qiu *et al.*, 2006).

Effects on heart failure

In the 1980's and 1990's, a number of well-designed trials were performed, showing that digoxin therapy increased the cardiac output by the left ventricle and thereby increased exercise time and decreased the symptoms of heart failure (GAXS, 1988; JAMA, 1988; DiBianco *et al.*, 1989; Gosselink *et al.*, 1997). Three extensive studies, Prospective Randomized Study of Ventricular Function and Efficacy of Digoxin (PROVED), Randomized Assessment of Digoxin on Inhibitors of the Angiotensin Converting Enzyme (RADIANCE), and Digitalis Investigators Group (DIG), played an important role in approving digoxin for the treatment of heart failure in 1998 by the Food and Drug Administration (Gheorghiade *et al.*, 2006). In the 12 week PROVED study, digoxin was randomly withdrawn from 113 patients with mild to moderate heart failure. These patients showed worsened exercise time and an increased incidence of treatment failure as compared to patients who continued to receive digoxin (Uretsky *et al.*, 1993). In 1993, Packer *et al.* conducted the RADIANCE study in which 178 patients with heart failure receiving digoxin, diuretics and angiotensin-converting-enzyme-inhibitors participated. Random withdrawal of digoxin worsened heart failure by 6 folds, deteriorated ejection fraction and exercise capacity while increasing heart rate and body weight (Packer *et al.*, 1993).

The Digitalis Investigation Group (DIG) performed in 1997 a double-blind clinical trial on 3397 patients with a 37 month follow-up. They showed that prescription of digoxin reduced hospitalization, whereas it did not influence mortality (DIG, 1997). Additionally, digoxin was shown effective in controlling the heart rate in atrial fibrillation (Fuster *et al.*, 2006). The effect of digoxin can be categorized in three groups: electrophysiological, hemodynamic and neurohormonal (Gheorghiade *et al.*, 2006).

The electrophysiological effect of digoxin can be divided into three effects: ionotropic, chronotropic, and dromotropic. The positive ionotropic effect is a consequence of the inhibitory effect on Na,K-ATPase, which increases the intracellular Na^+ concentration

leading to a rise in the intracellular Ca^{2+} concentration via the Na^{+}/Ca^{2+} exchanger and subsequent stimulation of heart muscle contraction (Hauptman & Kelly, 1999; Currie *et al.*, 2011).

The heart contains two nodes: the sinoatrial node (SA node) in the right atrium and the atrioventricular node (AV node) between the atria and ventricles of the heart. The impulses of the heart are generated in the SA node, spread through the atria where they activate the AV node, which in turn transfers the impulses from the atria to the ventricles. At therapeutic dosages, digoxin has a negative chronotropic effect, which is originated from its sympathoinhibitory effect. It decreases the ability of the heart to begin impulses due to external stimuli through the SA node; however, a supratherapeutic dose exerts a positive chronotropic effect and increases sinus rates and ultimately sinus arrest at very high dosages. In addition, a therapeutic dose of digoxin has a negative dromotropic effect and slows the conduction through the atrioventricular node, whereas it increases the conduction via this node in toxic dosages (Antman & Smith, 1985; Ferguson *et al.*, 1989; Hauptman & Kelly, 1999; Eichhorn & Gheorghiade, 2002). In general, automatic impulses are initiated after toxic dosages of digoxin, which are independent of the autonomic system of the heart.

Although digoxin was thought to be effective in heart failure treatment because of its inhibitory effect on Na,K-ATPase, it also has therapeutic hemodynamic and neurohormonal effects.

Digoxin increases heart contractility in both heart failure patients and healthy subjects. Baroreceptors adjust the blood pressure by influencing cardiac output and vascular smooth muscle tone via the central nervous system. In healthy subjects, baroreceptors function to increase systemic vascular resistance after digoxin-induced heart contractility, therefore, the cardiac output is not changed. As atrial baroreceptors are less sensitive to blood pressure changes in heart failure patients, increased contractility by digoxin results in a higher cardiac output (Gheorghiade & Ferguson, 1991; Eichhorn & Gheorghiade, 2002; Gheorghiade *et al.*, 2004). However, this is not observed in a healthy subject, because digoxin also increases the systemic vascular resistance (Ross *et al.*, 1960; Mason & Braunwald, 1964; Lee & Klaus, 1971; DeMots *et al.*, 1978; Vivo *et al.*, 2008). As no significant difference was observed between the expression of Na,K-ATPase in the heart of normal subjects and long term digoxin-administered patients (Braunwald, 1985; Schmidt *et al.*, 1991; Schmidt *et al.*, 1993; Eichhorn & Gheorghiade, 2002), the hemodynamic effect of digoxin seems to play a more important role than its electrophysiological effect to increase heart output.

Heart failure induces neurohormonal abnormalities via the renin-angiotensin system, adrenergic nervous system, cytokines, and vasopressin, which are all modulated in presence of digoxin. Therapeutic doses of digoxin restore the sensitivity of baroreceptors leading

to a reduction in sympathetic nervous system activity and therefore, decreased release of norepinephrine and renin in plasma (Gheorghiade & Ferguson, 1991; Eichhorn & Gheorghiade, 2002). In addition, digoxin-induced reduction of renin results in reduced aldosterone levels (Covit *et al.*, 1983; van Veldhuisen *et al.*, 1993; Gheorghiade *et al.*, 2004). Digoxin also has a vagomimetic effect due to its inhibitory effect on SA and AV conductions as described above (Ferguson *et al.*, 1989; Krum *et al.*, 1995).

Therapeutic dosing

Digoxin is preferably administered orally (tablet or elixir) or intravenously and not intramuscularly because it is painful (Smith *et al.*, 1984; Hanratty *et al.*, 2000). Doehrty *et al.* investigated the pharmacokinetic profile of digoxin administered via these three different routes. The increase of digoxin in serum was categorized as follows: intravenous > intramuscular > oral (elixir) > oral (tablet) (Doherty *et al.*, 1978; Smith *et al.*, 1984).

A beneficial effect of digoxin was observed among patients treated with less than 1 ng/ml compared with those treated with higher dosages (Packer *et al.*, 1993; Uretsky *et al.*, 1993; Slatton *et al.*, 1997). Moreover, patients with plasma digoxin levels between 0.5-0.9 ng/ml experienced reduced mortalities and hospitalization than those that used higher dosages (Ahmed *et al.*, 2006). Factors such as renal function, sex, age and other drugs that influence digoxin concentration in serum should be considered in administration of digoxin dosage. Generally, an oral dose of 0.125-0.250 mg digoxin per day is prescribed for a patient with heart failure to achieve a serum concentration of 0.5-1.0 ng/ml. In the case of atrial fibrillation, an intravenous loading dose of 0.25 mg every two hours is prescribed for rapid response and for maintenance therapy, an oral dose of 0.5 mg per day is prescribed (Packer *et al.*, 1993; Uretsky *et al.*, 1993; Adams *et al.*, 2002; Gheorghiade *et al.*, 2006; Vivo *et al.*, 2008).

Beside digoxin, digitoxin is prescribed in countries such as Germany, France, and Norway. The hydrophobicity of digitoxin enhances its gastrointestinal absorption. In addition, metabolism and excretion of digitoxin is different from digoxin (Roever *et al.*, 2000). Therapeutic concentrations of digitoxin in plasma should be between 10-30 ng/ml and a daily oral dose of 0.05-0.07 mg is recommended to achieve steady state within three days (Belz *et al.*, 2001). Ouabain, the hydrophilic DLC, has a short onset of action. Although ouabain is excreted mostly unchanged into urine, gastrointestinal excretion also occurs (Doherty *et al.*, 1978). Moreover, ouabain is not suitable for oral administration because of its poor gastrointestinal absorption (Joubert, 1990).

Toxicity

In 1785, Withering already described serious toxic effects of digitalis and different sensitivities of patients towards digitalis (Finch *et al.*, 1984). In 1969, a huge intoxication of digitoxin occurred in Juliana Hospital, Veenendaal, the Netherlands due to a production error in digoxin tablets. The tablets contained 0.2 mg digitoxin and 0.05 mg digoxin instead of 0.25 mg digoxin (Lely & van Enter, 1970). Beller *et al.* showed that of patients treated with digoxin, 20% had possible and definite digoxin toxicity, however, in 1997 the DIG group observed a lower toxicity incidence (Beller *et al.*, 1971; DIG, 1997). In a large heart failure study in 2006, it was reported that although the use of digoxin was declined from 31% in 2001 to 24% in 2004, the number of toxic exposures was not changed (Hussain *et al.*, 2006).

Generally, a plasma concentration of more than 2 ng/ml digoxin is considered toxic. DLCs toxicity symptoms vary from gastrointestinal indications to nervous system manifestations. Nausea, anorexia and fatigue are the most frequently observed and other symptoms include abdominal pain, diarrhea, dizziness, headache, muscular weakness, visual complaints such as flashing lights, halos and impairment in green-yellow perception (xanthopsia), psychic complaints and vomiting (Lely & van Enter, 1970; Rawlins, 1974; Eichhorn & Gheorghiade, 2002; Vivo *et al.*, 2008). The severity of toxicity depends on different factors. It has been reported that hyperthyroidism, hyperkalemia, childhood, and atrial fibrillation decrease digitalis toxicity, whereas hypokalemia, hypomagnesemia, hypercalcemia, hypoxia, ischemic heart disease, hypothyroidism and advanced age all aggravate toxicity (Vivo *et al.*, 2008). Most of the above-mentioned factors interfere with digitalis absorption, metabolism, and excretion, whose relation with digitalis toxicity is discussed in detail below.

Drug-drug interactions

The interactions of drugs with DLCs include pharmacodynamic as well as pharmacokinetic interactions at absorption, metabolism, and excretion levels (Table 2).

Absorption. In rat, guinea pig and human, DLCs are absorbed via passive diffusion into intestine (Greenberger *et al.*, 1969; Haass *et al.*, 1972; Smith *et al.*, 1984). Therefore, lipid-solubility of DLCs plays an important role in their absorption (Greenberger *et al.*, 1969; Shaw *et al.*, 1972; Lindenbaum, 1973). Gastrointestinal absorption of DLCs is influenced by food and the digestive system. The presence of food or fasting situation influences the concentration of digoxin when it is administered orally (White *et al.*,

1971). In addition, mucosal situation, intestinal mobility, malabsorption syndromes, gut microbial flora, and acidity of gastric fluid play a role in DLCs absorption. In an acidic condition, digoxin is easily hydrolyzed to digoxigenin and other metabolites with less toxicity (Gault *et al.*, 1980). In addition, drugs that alter digestive system-related factors can interact with DLC absorption. For example, drugs that increase intestinal mobility such as cathartics and drugs that bind to DLCs such as cholestyramine decrease DLCs' gastrointestinal absorption (Heizer *et al.*, 1971; Lindenbaum *et al.*, 1976; Kuhlmann *et al.*, 1981). Additionally, antacids (aluminum hydroxide, magnesium hydroxide and magnesium trisilicate) and cytostatic drugs (cyclophosphamide and bleomycin) decrease digoxin absorption by induction of diarrhea and intestinal damage, respectively (Kuhlmann *et al.*, 1981; Rodin & Johnson, 1988; Ehle *et al.*, 2011). Since digitoxin undergoes enterohepatic circulation, its absorption might also be influenced by drugs at this level, even when administered intravenously. For example, ion-exchange resins such as cholestyramine and colestipol hydrochloride decrease enterohepatic circulation of digitoxin and consequently its absorption (Bazzano & Bazzano, 1972; Smith *et al.*, 1984). In Table 2, drugs that are interacting with DLCs at different levels are summarized. In addition to passive diffusion, intestinal efflux transporters such as P-glycoprotein have an important role in limiting the bioavailability of digoxin. This will be discussed further in Excretion section.

Metabolism. Factors that can influence DLCs metabolism vary based on the type of DLCs and its metabolic route. Less than 1% of administered digoxin is metabolized to digoxigenin, digoxigenin mono-digitoxose and digoxigenin bis-digitoxose (Doherty, 1968). However, in 10% of digoxin-using patients, digoxin is metabolized by *Eggerthella lenta* (classified as *Eubacterium lentum*) in gut and metabolites such as dihydrodigoxin and dihydrodigoxigenin were observed in the urine (Lindenbaum *et al.*, 1981; Saha *et al.*, 1983; Smith *et al.*, 1984; Robertson *et al.*, 1986; Moffett *et al.*, 2013).

Recently, Haiser *et al*. has shown that two gene operons in *E. lenta DSM2243* are highly up-regulated upon exposure to digoxin. The protein products of these operons are responsible for reduction of α,β-unsaturated butyrolactone ring of cardenolides such as digoxin. Interestingly, arginine suppresses these operons and therefore, high protein diet decreased digoxin reduction and increased digoxin levels in serum and urine of mice. This highlights the role of gut bacterium and patients diet on pharmacokinetics of DLCs (Haiser *et al.*, 2013; Haiser *et al.*, 2014).

In addition, the concentrations of bacterium-produced metabolites were reduced after co-administration of antibiotics such as erythromycin, clarithromycin, roxithromycin, and tetracycline (Luchi & Gruber, 1968; Peters *et al.*, 1978; Lindenbaum *et al.*, 1981; Bizjak & Mauro, 1997; Nawarskas *et al.*, 1997). However, a number of these antibiotics

influence digoxin excretion and thereby plasma levels, which is further discussed below. In rat, digoxin is metabolized by cytochrome P450 and excreted into bile as didigitoxide, monodigitoxide, and digoxigenin (Salphati & Benet, 1999).

Unlike digoxin, digitoxin is extensively metabolized in the liver and there are many drugs that influence its metabolism. During digitoxin metabolism, first, the three sugar groups are removed leading to bisdigitoxide, monodigitoxide, and finally digitoxigenin. Digitoxigenin and digoxigenin can be deactivated after epimerization from 3β-OH to 3α-OH, which can be further metabolized to glucuronide or sulfate derivatives (Herrmann & Repke, 1964; Abshagen & Rietbrock, 1973). For the β to α epimerization, first the hydroxyl group of digitoxigenin and digoxigenin is converted to a ketone group by human liver alcohol dehydrogenase (Frey & Vallee, 1980).

Digitoxin and digitoxigenin are further metabolized by the addition of a hydroxyl group at position 12 that converts digitoxin and digitoxigenin to digoxin and digoxigenin, respectively (Solomon & Abrams, 1972). Moreover, *in vitro* studies have shown that C5 and C6 hydroxylation of digitoxigenin leads to more polar digitoxigenin metabolites, which are renally excreted (Bulger & Stohs, 1973; Bulger *et al.*, 1974). The same procedures of epimerization can occur for digoxigenin. Drugs such as diphenylhydantoin, phenylbutazone, phenobarbital, rifampicin and spironolactone decrease plasma digitoxin levels, due to induction of its hepatic metabolism (Peters *et al.*, 1974; Vohringer *et al.*, 1975; Wirth *et al.*, 1976; Boman *et al.*, 1980; Poor *et al.*, 1983) (Table 2).

Table 2. Pharmacokinetic and Pharmacodynamic drug-drug interactions of digitalis-like compounds

DDIs [a]	Drug name	Drug function	Mechanism of interaction	Effect on digitalis	References
Pharmacokinetic					
Absorption level	Cyclophosphamide	cytostatic	damages to intestinal mucosa	↓β-acetyl digoxin, ↓digoxin absorption	(Kuhlmann *et al.*, 1981)
	Bleomycin	*	*	↓digoxin absorption	(Rodin & Johnson, 1988)
	Propantheline	*	↓gastrointestinal motility	↑digoxin absorption	(Manninen *et al.*, 1971)
	Cholestyramine	ion exchange resin	binds to digoxin	↓digoxin absorption	(Brown *et al.*, 1978)
	Colestipol	*	binds to digitoxin	↓digitoxin absorption	(Bazzano & Bazzano, 1972)
	p-Cresol	uremic toxins	↓hepatic uptake of digoxin	↓digitoxin absorption	(Tsujimoto *et al.*, 2008b)
	Neomycin	antibiotic	↑intestinal malabsorption	↓digoxin absorption	(Ratnaike & Jones, 1998)
	Sulfasalazine	*	?	↓digoxin absorption	(Juhl *et al.*, 1976)
	Phenytoin	antiepileptic	?	↓digoxin absorption	(Ehle *et al.*, 2011)
	Penicillamine	Immunosuppressive	Induction of diarrhea	↓digoxin absorption	(Ratnaike & Jones, 1998)
	Aluminium hydroxide	antacid	Induction of diarrhea	↓digoxin absorption	(Rodin & Johnson, 1988)
	Magnesium hydroxide	*	*	*	
	Magnesium trisilicate	*	*	*	
Metabolism level	Erythromycin	antibiotic	destroys gut flora, ↓digoxin metabolism	↑digoxin of plasma	(Lindenbaum *et al.*, 1981)
	Tetracycline	*	*	*	(Lindenbaum *et al.*, 1981)
	Clarithromycin	*	*	*	(Nawarskas *et al.*, 1997)
	Azithromycin	*	destroys gut flora, ↓digitoxin metabolism	↑digitoxin of plasma	(Thalhammer *et al.*, 1998)
	Phenylbutazone	NSAIDs [b]	↑digitoxin hepatic metabolism	↓digitoxin of plasma	(Solomon & Abrams, 1972)
	Diphenylhydantoin	antiepileptic	*	*	
	Phenobarbital	*	*	*	
	Rifampicin	antibiotic	↑digitoxin metabolism	↓digitoxin of plasma	(Boman *et al.*, 1980)
	Rifampin	*	↑digitoxin metabolism	↓digitoxin of plasma	(Poor *et al.*, 1983)
	Spironolactone	antimineralocorticoid	↑digitoxin metabolism	↓digitoxin of plasma	(Wirth *et al.*, 1976)
	Probenecid	uricosuric	↓digitoxin metabolism in rat	↑digitoxin of plasma	(Damm & Erttmann, 1975)

[a] DDIs: Drug-drug interactions, [b] NSAIDs: Nonsteroidal anti-inflammatory drugs, * Refer to drug function, mechanism of interaction, effect or reference of the previous row.

DDIs	Drug name	Drug function	Mechanism of interaction	Effect on digitalis	References
Excretion level	Rifampin	antibiotic	Induction of intestinal P-gp, ↑excretion	↓digitoxin of plasma	(Greiner *et al.*, 1999)
	Guanethidine	antihypertensive	↓glomerular filtration	↓digoxin renal excretion	(Smith *et al.*, 1984)
	Bethanidine	*	*	*	*
	Debrisoquine	*	*	*	*
	Quinidine	antiarrhythmic agent	Inhibition of P-gp, ↓excretion	↑digoxin and digitoxin of plasma	(Fromm *et al.*, 1999)
	Verapamil	*	*	↓digoxin and methyl-digoxin excretion	(Ito *et al.*, 1993a; Laer *et al.*, 1998; Verschraagen *et al.*, 1999)
	Amiodarone	*	*	↓digoxin and digitoxin excretion	(Ito *et al.*, 1993b; Laer *et al.*, 1998)
	Propafenone	*	*	↓digoxin and digitoxin excretion	(Sphakianaki *et al.*, 1992; Woodland *et al.*, 1997)
	Mexiletine	*	*	↓digitoxin excretion	(Sphakianaki *et al.*, 1992)
	Diltiazem	*	*	↓digitoxin excretion	(Kuhlmann, 1985)
	Clarithromycin	antibiotic	*	↓digoxin excretion	(Hughes & Crowe, 2010)
	Roxithromycin	*	*	↓digoxin excretion	*
	Erythromycin	*	*	↓digoxigenin excretion	*
	Ciprofloxacin	*	*	↓digoxin excretion	(Moffett *et al.*, 2013)
	Rifampin	*	Inhibition of intestinal P-gp, ↑absorption	↑digoxin and digitoxin of plasma	(Reitman *et al.*, 2011)
	Telithromycin	*	Inhibition of intestinal and renal P-gp	↑absorption, ↓excretion of digoxin	(Nenciu *et al.*, 2006)
	Ketoconazole	antifungal	Inhibition of P-gp, ↓excretion	↓digoxin excretion	(Fan & Rodriguez-Proteau, 2008)
	Tryptanthrin	*	*	*	(Zhu *et al.*, 2011)
	Itraconazole	*	Inhibition of intestinal P-gp, ↑absorption	↑digoxin of plasma	(Takara *et al.*, 1999)
	Omeprazole	proton pump inhibitor	Inhibition of P-gp	↓digoxin excretion	(Li *et al.*, 2013)
	Pantoprazole	*	*	*	(Pauli-Magnus *et al.*, 2001b)
	Lansoprazole	*	*	*	(Pauli-Magnus *et al.*, 2001b)
	Lumefantrine	antimalarial	Inhibition of P-gp, ↓excretion	↓digoxin excretion	(Oga *et al.*, 2012)
	Amodiaquine	*	*	*	*
	Artesunate	*	*	*	*
	Doxazosin	antihypertensive	Inhibition of P-gp, ↓excretion	↑digoxin of plasma	(Takara *et al.*, 2002)

DDIs	Drug name	Drug function	Mechanism of interaction	Effect on digitalis	References
	Artesunate	*	*	*	(Takara *et al.*, 2002)
	Doxazosin	antihypertensive	Inhibition of P-gp, ↓excretion	↑digoxin of plasma	*
	Spironolactone	potassium-sparing diuretic	Inhibition of P-gp, ↓excretion	↑plasma digoxin and digitoxin	(Steiness, 1974; Carruthers & Dujovne, 1980)
	Triamterene	*	*	↑digoxin of plasma	(Waldorff *et al.*, 1983)
	Cyclosporine A	immunosuppressant	*	*	(Okamura *et al.*, 1993)
	Atorvastatin	statins	*	*	(Boyd *et al.*, 2000)
	Mibefradil	calcium channel blocker	*	*	(Wandel *et al.*, 2000)
	Acetaminophen	pain reliever	Inhibition of intestinal P-gp, ↑absorption	↑digitoxin of plasma	(Novak *et al.*, 2013)
	Sertraline	serotonin reuptake inhibitor	Inhibition of blood-brain barrier P-gp	↑digoxin accumulation	(Kapoor *et al.*, 2013)
	Rilpivirine	HIV treatment	Inhibition of P-gp, ↓excretion	↑digoxin of plasma	(Moss *et al.*, 2013)
	Mifepristone	abortifacient	*	*	(Woodland *et al.*, 2003)
	Carvedilol	β-adrenoceptor antagonists	Inhibition of intestinal P-gp, ↑absorption	↑digitoxin of plasma	(Aiba *et al.*, 2005)
	Pimobendan	calcium sensitizer	*	*	
	Propranolol	β-adrenoceptor antagonists	Inhibition of P-gp	↑digitoxin of plasma	(Bachmakov *et al.*, 2006)
	Azelastine	histamine antagonist	Inhibition of hepatic and intestinal P-gp	↑digoxin absorption, ↓excretion	(Katoh *et al.*, 2001)
	Dipyridamole	antiplatelet	Inhibition of intestinal P-gp, ↑absorption	↑digitoxin of plasma	(Verstuyft *et al.*, 2003)
	Indomethacin	NSAIDs	Inhibition of P-gp, ↓excretion	↑digoxin of plasma	(Jorgensen *et al.*, 1991; Verstuyft *et al.*, 2003; Zrieki *et al.*, 2008)
	Diclofenac	*			(Davies & Anderson, 1997; Awara *et al.*, 2004)
	Aminoglutethimide	anti-steroid	Induction hepatic enzymes	↑digitoxin excretion	(Lonning *et al.*, 1984)
	Bupropion	antidepressant	Induction of OATP4C1	↑digoxin excretion	(He *et al.*, 2014)
Pharmacodynamic	Hypokalemia		↑cardiac digoxin, ↓digoxin excretion	Plasma digoxin fluctuation	(Steiness, 1978; Vivo *et al.*, 2008)
Na,K-ATPase	Hypomagnesemia		induction of hypokalemia	Plasma digoxin fluctuation	(Neff *et al.*, 1972; Vivo *et al.*, 2008)

Furthermore, the metabolic routes of other DLCs, which are not often applied in the clinic, have been clarified. Ouabain is weakly absorbed after oral administration (1.4% of dose), and it is mainly excreted via the kidney, 80% of which in the unchanged form and 20% as ouabain metabolites (Strobach *et al.*, 1986). Cymarin is metabolized via different steps in the liver: cleavage of its sugar (cymarose) to K-strophanthidin, reduction of the aldehyde group at position C19 and subsequent conversion of cymarin to cymarol and K-strophanthidin to K-strophanthidol, and formation of conjugated metabolites at C3 position (glucuronide and sulphate) (Moerman, 1965; Strobach *et al.*, 1986). In addition, the cleavage of K-strophanthoside sugar moiety by intestinal flora can lead to cymarin production, which is metabolized further according to the above-mentioned steps. Sometimes the metabolism of DLCs depends on their route of administration. K-strophanthoside is metabolized mainly to glucuronates and sulphates when it is administered orally; on the other hand, it is excreted unchanged in the case of intravenous administration (Strobach *et al.*, 1986). Furthermore, proscillaridin A and its synthetic form, methyl-proscillaridin are metabolized in the liver. The aglycone of these two DLCs, 3β-scillarenin, its epimer 3α-scillarenin, and 12β-OH-episcillarenin are the metabolites of these DLCs found in human urine and plasma (Belz *et al.*, 1974; Rietbrock & Staud, 1975).

Excretion. Cardiac glycosides and their metabolites can be eliminated via feces by intestinal and biliary excretion and via urine by renal excretion. The elimination half-life of digoxin is 30-40 hours. After one week of continuous administration, a steady-state concentration in plasma is achieved (Hanratty *et al.*, 2000). About 60-80% of bioavailable digoxin is excreted unchanged into urine, by glomerular filtration and active tubular secretion (Sumner & Russell, 1976; Hanratty *et al.*, 2000). A small amount is metabolized in the liver and the rest is secreted via the intestine. Drugs that affect glomerular filtration can influence renal DLC excretion. For example, antihypertensive agents such as guanethidine, bethanidine, and debrisoquine delay digoxin excretion due to reduction of glomerular filtration rate (Smith *et al.*, 1984). Besides glomerular filtration, tubular secretion plays a role in digoxin excretion and can be influenced by drugs such as spironolactone and triamterene that reduce digoxin renal elimination by inhibition of P-gp whose role in digoxin excretion is discussed below (Steiness, 1974; Waldorff *et al.*, 1983; Nakamura *et al.*, 2001; Varma *et al.*, 2003).

Many studies have demonstrated that co-administration of digoxin with drugs such as quinidine and verapamil reduces digoxin clearance and distribution (Ejvinsson, 1978; Doering, 1979; Reiffel *et al.*, 1979; Klein *et al.*, 1980; Pedersen *et al.*, 1981; Schwartz *et al.*, 1982). In 1992, digoxin was reported to be a P-glycoprotein (P-gp) substrate by De Lannoy *et al*. (de Lannoy & Silverman, 1992).

P-gp belongs to the ATP-binding cassette transporter (ABC) family and is mainly expressed in kidney, intestine, liver, and brain (Sakaeda *et al.*, 2002; Choudhuri & Klaassen, 2006). An *in vivo* study using *mdr1a* (-/-) knockout mice showed that the absence of P-gp increased digoxin accumulation in the brain 20-50 times compared to wild type mice and decreased the digoxin elimination rate (Schinkel *et al.*, 1995). P-gp is prominently expressed in the brush border of renal proximal tubules, the canalicular membrane of hepatocytes, luminal membrane of capillary endothelial cells of brain and testis, the apical membrane of enterocytes, and in placental trophoblasts and adrenal gland (Thiebaut *et al.*, 1987; Sugawara *et al.*, 1988; Cordon-Cardo *et al.*, 1989). This tissue distribution of P-gp guarantees the excretion of potential toxic compounds like digoxin from the body into bile and urine and prevents its accumulation in brain and testis. Furthermore, P-gp transports digoxigenin, as digoxin metabolite, acetyl-digoxin, methyl-digoxin, and digitoxin (Pauli-Magnus *et al.*, 2001a) (Table 3).

Discovery of P-gp as digoxin transporter elucidated the mechanism of many digoxin-drug interactions. The concomitant administration of quinidine and digoxin increases the digoxin plasma concentration due to inhibition of P-gp by quinidine (Ito *et al.*, 1993a; Fromm *et al.*, 1999). In addition, quinidine decreases biliary excretion of digoxin by 40% (Angelin *et al.*, 1987). Generally, drugs that are a substrate or inhibitor of P-gp can influence active renal, biliary, and intestinal excretion of digoxin and induce its toxicity. Additionally, P-gp plays an important role in intestinal absorption of digoxin and its bioavailability. The intestinal expression of P-gp is associated negatively with the plasma concentration of orally administered digoxin but not intravenously administered digoxin (Greiner *et al.*, 1999). For example, rifampicin has been shown to induce the expression of intestinal P-gp. Co-administration of rifampicin leads to reduction of orally-administered digoxin absorption with no significant effect on renal digoxin excretion (Greiner *et al.*, 1999; Finch *et al.*, 2002). P-gp expression is induced by rifampicin via the nuclear pregnane X receptor (PXR) (Drescher *et al.*, 2003; Larsen *et al.*, 2007). The presence of PXR in the intestine but not in the kidney could explain the lack of rifampicin-induced P-gp expression in the kidney (Lehmann *et al.*, 1998; Kullak-Ublick & Becker, 2003). On the other hand, rifampicin is an acute inhibitor of P-gp and it seems that the overall rifampicin effect is a combination of inhibition and induction of P-gp (Reitman *et al.*, 2011).

As is listed in Table 2, different groups of drugs such as antiarrhythmic agents (verapamil, amiodarone, propafenone and mexiletine) (De Cesaris *et al.*, 1983a; b; Sphakianaki *et al.*, 1992; Ito *et al.*, 1993b; Woodland *et al.*, 1997; Laer *et al.*, 1998; Verschraagen *et al.*, 1999), antibiotics (clarithromycin, roxithromycin, ciprofloxacin, rifampin and telithromycin) (Nenciu *et al.*, 2006; Hughes & Crowe, 2010; Reitman *et al.*, 2011; Moffett *et al.*, 2013), antifungal agents (ketoconazole, tryptanthrin,

itraconazole) (Partanen *et al.*, 1996; Fan & Rodriguez-Proteau, 2008; Zhu *et al.*, 2011), anti-malarial medicines (lumefantrine, amodiaquine, and artesunate) (Oga *et al.*, 2012) and proton pump inhibitors (omeprazole, pantoprazole and lansoprazole) (Pauli-Magnus *et al.*, 2001b; Li *et al.*, 2013) decrease digoxin and digitoxin excretion due to P-gp inhibition and increase their oral absorption. Interestingly, not only drugs that are P-gp inhibitors can modulate the digoxin concentration and lead to DDIs, but also metabolites of administered drugs. Kotah *et al*. showed that azelastine and amiodarone and their metabolites are P-gp inhibitors and thus influence digoxin kinetics (Katoh *et al.*, 2001). Propafenone and its two metabolites, 5-hydroxy propafenone and N-desalkyl propafenone, are inhibitors of P-gp-mediated digoxin transport (Bachmakov *et al.*, 2005). Drugs that inhibit P-gp and thereby decrease excretion of digoxigenin, digoxin, methyl-digoxin, and digitoxin have been listed in Table 2.

Daily food ingredients contain compounds that modulate P-gp activity or expression and they could lead to variability in digoxin concentration. Capsaicin, the ingredient of chili peppers, can reduce digoxin plasma levels by increasing expression of P-gp. In addition, major ingredients of turmeric powder, ginger and black pepper, curcumin, 6-gingerol and piperine, respectively, inhibit P-gp-mediated digoxin transport (Han *et al.*, 2006; Han *et al.*, 2008; Zhang & Lim, 2008; Yue *et al.*, 2012). Further, flavonoids, present in citrus fruit, have been shown to inhibit P-gp and modulate digoxin transport (Yoo *et al.*, 2007).

Besides DDIs, other factors can change expression and function of P-gp such as mutations or polymorphisms might influence digoxin excretion and toxicity. Hoffmeyer *et al.* showed that individuals who are homozygous for a polymorphism in exon 26 of P-gp (C3435T) have lower expression and function of P-gp and consequently higher digoxin plasma levels due to increased absorption and reduced excretion (Hoffmeyer *et al.*, 2000), however, there are also populations in which the digoxin concentration was not influenced by this polymorphism (Sakaeda *et al.*, 2001; Gerloff *et al.*, 2002; Morita *et al.*, 2003).

OATP4C1, an organic anion transporting polypeptide (OATP) family member that is expressed in the kidney, was found to be a digoxin and ouabain uptake transporter. OATP4C1 located in the basolateral membrane of proximal tubular cells plays a role in the uptake of digoxin and ouabain from blood (Mikkaichi *et al.*, 2004). Recently, bupropion, an antidepressant drug, has been found to stimulate human OATP4C1-mediated digoxin transport *in vitro* (He *et al*., 2014). Human Multiple drug resistance 3 protein (MDR3), a phosphatidylcholine translocase, is expressed in the liver and has been reported as digoxin transporter *in vitro*; however, no DDIs for MDR3 are known (Smith *et al.*, 2000).

DDIs can occur at the level of active uptake of drugs into the liver followed by biliary excretion. The hepatic solute carrier (SLC) transporters play an important role in the absorption of drugs from the blood. The uptake of ouabain by rat liver was found to be transporter-dependent and was inhibited by digitoxin, whose uptake is by simple diffusion (Schwenk, 1980; Schwenk *et al.*, 1981). In rat, Oatp2, expressed in brain, liver and kidney, is responsible for digoxin and ouabain uptake (Noe *et al.*, 1997; Reichel *et al.*, 1999). Ouabain is also found to be transported by Oatp1 in mouse (Hagenbuch *et al.*, 2000). Therefore drugs, that inhibit active uptake of digoxin or ouabain in rat liver, cause DDI at uptake level. Rifampicin and amiodarone inhibit Oatp2-mediated uptake of digoxin in rat and consequently increases digoxin plasma levels (Lau *et al.*, 2004; Funakoshi *et al.*, 2005). Kullak-Ublik *et al.* reported OATP1B3 (OATP8) and OATP1A2 (OATP-A) as human transporters of digoxin and ouabain, respectively. Their involvement in DDIs is not yet clear and need to be studied in more details (Kullak-Ublick *et al.*, 2001). The uptake and efflux transporters of DLCs have been listed in Table 3.

Since digoxin and digitoxin are most often applied in therapy, DDIs for other DLCs have not been studied; however, their route of excretion has been determined. Ouabain, cymarin, k-strophanthoside and their metabolites cymarol, strophanthidin, strophanthidol, helveticoside and helveticosol are mainly excreted by the kidney (Strobach *et al.*, 1986). Furthermore, peruvoside is eliminated by renal and hepatic excretion; therefore, renal excretion is significantly increased in patients with cirrhosis to compensate for impaired biliary excretion (Lahrtz & van Zwieten, 1968). Elimination of proscillaridin A is by extrarenal processes. The conjugates of proscillaridin A are eliminated via the bile or reabsorbed by enterohepatic recycling after deconjugation (Andersson *et al.*, 1977b; Andersson *et al.*, 1977c). Although the excretion routes of many DLCs have been determined, information about their possible excretory transporters that could be involved in DDIs is lacking.

Pharmacodynamic interactions

Binding to cardiac Na,K-ATPase can be another site for the interaction of drugs with DLCs. DLCs have a higher affinity for myocardial than skeletal muscle Na,K-ATPase. As DLCs and potassium compete for binding to Na,K-ATPase and DLCs bind without potassium ion (Palasis *et al.*, 1996), hypokalemia increases DLCs binding to cardiac Na,K-ATPase and its toxicity (Hall *et al.*, 1977; Steiness, 1978). Consequently, higher and more variable plasma concentrations of DLCs are observed during hypokalemia and hypomagnesemia (Dutta *et al.*, 1968; Vivo *et al.*, 2008) (Table 2). Therefore, hyperkalemia and hypokalemia induced by diuretics, potassium-depleting diet, corticosteroid therapy, and insulin therapy can predispose digoxin-administered patients in toxicity (Smith *et al.*, 1984).

As mentioned above, digoxin has a negative dromotropic effect and it reduces the conduction through the atrioventricular node. Therefore, concomitant administration of digoxin with catecholamine-depleting drugs like β-adrenergic blockers leads to bradycardia and atrioventricular block (Lown *et al.*, 1961; Smith *et al.*, 1984; Vincent *et al.*, 1984; Hanratty *et al.*, 2000; Vivo *et al.*, 2008).

Predisposing factors in toxicity

Although DDIs play a key role in digoxin toxicity, the altered physiological conditions and diseases could also predispose digoxin treated patients to a higher risk of toxicity. As aging can coexist with renal insufficiency and kidney diseases, it may indirectly affect digoxin excretion and increases the risk of toxicity in elderly patients (Vivo *et al.*, 2008). Aging and its consequences can influence absorption, metabolism, and excretion of digoxin which is associated with a higher possibility of digoxin toxicity. Elderly people have a slower rate of digoxin absorption (Cusack *et al.*, 1979; Hammerlein *et al.*, 1998) which can be caused by a relatively high gastric pH and low gastrointestinal motility. As digoxin binds to skeletal muscle rather than other tissues, aging that is associated with less body mass, can influence the volume of digoxin distribution and increase the risk of toxicity (Cusack *et al.*, 1979; Haas & Young, 1999).

As DLCs are absorbed by the gastrointestinal tract, malabsorption syndromes and diseases that associate with high gastrointestinal pH, low motility and changed intestinal microflora, small bowel transit and short intestine length alter digoxin absorption (Ehrenpreis *et al.*, 1994; Hanratty *et al.*, 2000; Severijnen *et al.*, 2004; Ward, 2010). Digoxin has a large volume of distribution (4-7 L/Kg) and its body distribution can be affected by body mass and adipose tissue. In the condition of decreased lean body mass and increased adipose tissue, which occurs often during aging, the volume of distribution for hydrophilic drugs is reduced and for lipophilic drugs increased. Thus, the body mass and obesity of the patients should be considered in DLCs administration (Cusack *et al.*, 1979; Abernethy *et al.*, 1981; Hanratty *et al.*, 2000).

Diseases such as hyperthyroidism, hypoglycemia, diabetes mellitus, and respiratory diseases influence DLCs excretion indirectly as well. For example, hypoglycemia has shown to down-regulate P-gp expression and impair digoxin excretion. Whereas, hyperthyroidism enhances P-gp expression and decreases the plasma digoxin concentration significantly (Nishio *et al.*, 2008; Vivo *et al.*, 2008; Yeh *et al.*, 2012).

Table 3. Influx and efflux transporters of DLCs

Transporters	Substrate	Species	Organs	References
Oatp1	ouabain	mouse	liver, kidney	(Hagenbuch *et al.*, 2000)
Oatp1a4	digoxin, ouabain	rat	brain, liver, kidney	(Hagenbuch *et al.*, 2000)
				(Noe *et al.*, 1997)
				(Reichel *et al.*, 1999)
Oatp4c1	digoxin	rat	kidney	(Mikkaichi *et al.*, 2004)
OATP1B3	digoxin	human	liver	(Kullak-Ublick *et al.*, 2001)
OAP1A2	ouabain		liver	(Kullak-Ublick *et al.*, 2001)
OATP4C1	digoxin, ouabain		kidney	(Mikkaichi *et al.*, 2004)
P-glycoprotein	digoxin	human, mouse	brain, liver, kidney	(de Lannoy & Silverman, 1992)
				(Schinkel *et al.*, 1995)
P-glycoprotein	digitoxin	human	intestine, placenta	(Pauli-Magnus *et al.*, 2001a)
P-glycoprotein	methyl-digoxin			(Pauli-Magnus *et al.*, 2001a)
P-glycoprotein	acetyl-digoxin			(Pauli-Magnus *et al.*, 2001a)
P-glycoprotein	digoxigenin			(Hughes & Crowe, 2010)
MDR3 P-gp	digoxin			(Smith *et al.*, 2000)

Therefore, in hyperthyroid condition, increased digoxin and digitoxin doses are needed for therapeutic purposes (Frye & Braunwald, 1961; Croxson & Ibbertson, 1975; Curfman *et al.*, 1977). The necessary concentration of digoxin to control the ventricular rate in patients with hypoxia (resulting from pulmonary disease) is higher (2.5-6 ng/ml) than the therapeutic dosage (Goldman *et al.*, 1975). It was shown that P-gp expression is induced by ambient hypoxia *in vitro*, which might explain the requirement for the higher digoxin dose in patients with hypoxia (Comerford *et al.*, 2002).

Toxicity treatment

In the case of DLCs toxicity, oxygen supplementation is applied to control hypoxia. In addition, atropine and lidocaine are used for symptomatic bradycardia and ventricular tachycardia, respectively (Vivo *et al.*, 2008). Atropine suppresses the inhibitory effect of digoxin on heart SA and AV nodes and lidocaine enhances ventricular automaticity. Inductions of vomiting and gastric lavage are not recommended because they both increase vagal tone and may worsen arrhythmias (Dugdale, 2013; Vinod & James, 2014). Hydration with IV fluids such as saline and sometimes correction of electrolyte imbalance could be performed as supportive care and it should also be considered that correction of electrolyte imbalances may cause dysrhythmias. Toxicity-induced hyperkalemia could

be controlled using sodium bicarbonate to correct metabolic acidosis (Vinod & James, 2014). Insulin plus glucose improves potassium uptake by cells and magnesium, as the regulating cofactor of Na,K-ATPase, could be used to control hyperkalemia (Reisdorff *et al.*, 1986; Ahee & Crowe, 2000). Magnesium reverses digitalis-induced inhibition of Na,K-ATPase, blocks cellular Ca^{2+} influx, reduces plasma potassium levels and stabilizes myocardial conductivity; however, extra caution should be considered for treatment of digitalis toxicity in patients with impaired renal function who might already have hypermagnesemia (Beller *et al.*, 1974; Rajapakse, 2009). Additionally, charcoal and cholestyramine (discussed in DDIs section) can be used to interrupt DLCs absorption and enterohepatic circulation (Lalonde *et al.*, 1985; Park *et al.*, 1985; Rawashdeh *et al.*, 1993).

Digibind and Digifab are available forms of digoxin IgG antibody developed in sheep to treat life threatening digoxin toxicity. Due to the high affinity of these antibodies digoxin redistributes from tissues and digoxin-antibody complex will be excreted via the kidneys (Bauman *et al.*, 2006; Vivo *et al.*, 2008). Antibody treatment is recommended in the case of massive ingestion of digitalis, at digoxin concentrations higher than 10 ng/ml and hyperkalemia (serum potassium higher than 5 mEq/L) (Yang *et al.*, 2012).

Endogenous digitalis-like compounds

The conserved site in Na,K-ATPase for binding of DLCs and existence of bufadienolide in some amphibians led to the speculation that DLCs might be produced in the human body too. In 1991, an ouabain-like compound (10 μg) was isolated from 85 liters human plasma (Hamlyn *et al.*, 1991). Later on, ouabain-like factors were also measured in dog plasma, bovine hypothalamus and adrenal glands of rat and human (Haupert & Sancho, 1979; Tymiak *et al.*, 1993; Schneider *et al.*, 1998; Kawamura *et al.*, 1999). Beside ouabain-like factors, other DLCs such as digoxin, bufalin, marinobufagenin, proscillaridin A and 19-norbufalin have been found in human plasma, bile, urine, cataractous lens, placenta and hypothalamus (Diamandis *et al.*, 1985; Kieval *et al.*, 1988; Goto *et al.*, 1991; Lichtstein *et al.*, 1993; Hilton *et al.*, 1996). It was shown that the ouabain concentration is elevated in conditions such as sodium imbalance, chronic renal failure, hyperaldosteronism, congestive heart failure, and preeclampsia (Kelly *et al.*, 1986; Masugi *et al.*, 1986; Masugi *et al.*, 1987; Gottlieb *et al.*, 1992; Averina *et al.*, 2006). Moreover, the concentration of endogenous ouabain was associated with high blood pressure, vasoconstriction, and thickness of the left ventricular heart wall in human. Interestingly, these effects have not been observed with other DLCs such as digoxin and digitoxin (Manunta *et al.*, 2000; Pierdomenico *et al.*, 2001). Two mechanisms

have been postulated by which ouabain is involved in hypertension: 1) Inhibition of vascular α2 Na,K-ATPase and increased peripheral vascular resistance 2) Increased re-absorption of renal Na^+ and volume expansion due to inhibition of α1 Na,K-ATPase in renal caveolae (Bagrov *et al.*, 2009). Beside all studies trying to measure endogenous DLCs, there were also reports on failure to detect endogenous ouabain in human plasma using ultra-sensitive methods (Doris *et al.*, 1994; Lewis *et al.*, 1994; Nicholls *et al.*, 2009; Baecher *et al.*, 2014).

The detection and isolation of bufadienolides in mammalian tissues arouse controversy about the biosynthesis of these compounds. As DLCs have steroid rings in their structure, the first precursor which was suggested to be cholesterol. Applying radiolabeled cholesterol and acetate, the biosynthesis of digoxin and marinobufagenin in murine adrenal cells was demonstrated *in vitro* (Siperstein et al., 1957; Dmitrieva et al., 2000; Qazzaz et al., 2004). If endogenous DLCs originated from steroids, enzymes for its synthesis have to be present. Enzymes for the conversion of the cholesterol side chain to the DLC lactone ring have not been described. Moreover, the structure of steroid rings in mammals is in *trans-trans-trans* form, however, DLCs have *cis-trans-cis* steroid rings (Qazzaz *et al.*, 2004).

Signal transduction by digitalis-like compounds

Interestingly, very low DLC concentrations that do not inhibit Na,K-ATPase transduce signaling pathways which contribute to cell growth and apoptosis (Prassas & Diamandis, 2008). Localization of Na,K-ATPase in caveolae, which are enriched in several signaling molecules, suggests a signal transduction pathway due to DLC binding to Na,K-ATPase. One of these signaling molecules associated with caveolae is Src, which binds to the cytosolic part of Na,K-ATPase. The binding of DLCs such as ouabain to the α-subunit induces Src activation and release (Tian *et al.*, 2006; Riganti *et al.*, 2011). Next, activated Src induces various signaling cascades. It may trans-activate epidermal growth factor receptor (EGFR) which leads to the Src/Ras/Raf/ERKs pathway (Wang *et al.*, 2004). In addition, Src increases the hydrolysis of phosphatidylinositol-4,5-bisphosphate due to phosphorylation and activation of phospholipase-γ, which opens the inositol 1, 4, 5-triphosphate (IP3) receptor/Ca^{2+} channel and increases the cytosolic Ca^{2+} concentration due to release of calcium from its intracellular storage. This increased Ca^{2+} concentration does not depend on Na^+/Ca^{2+} exchanger activity (Yuan *et al.*, 2005). The resulting Ca^{2+} oscillations induce cell proliferation by activating transcription factors such as AP1 (activating protein 1) and NF-κB (nuclear factor-κB) (Miyakawa-Naito *et al.*, 2003). The activation of ERK1/2 and c-Jun NH_2-terminal kinase (JNK) followed by Src activation results in increased cell cycle inhibitors and reduction of cell proliferation.

The pro-apoptotic effect of DLCs due to their influence on molecules such as Bad, Bcl-2, $p21^{cip1}$ and Bax has been reported in different cell types such as human breast cancer cells, human prostate cancer cells and human lung cancer cells (Riganti *et al.*, 2011). Kometiani *et al.* demonstrated that non-inhibiting concentrations of ouabain and therapeutic concentrations of digoxin and digitoxin inhibit human breast cancer cell proliferation due to activation of Src, EGFR1/ 2, $p21^{cip1}$ and JNK (Kometiani *et al.*, 2005). The effect of DLCs such as digoxin and ouabain on down-regulation of P53 via MAP/ERK were reported by Wang et al. (Wang *et al.*, 2009). The signaling cascades induced by DLCs may result in cell migration, apoptosis, impaired cell metabolism and altered gene expression, thus, it seems that DLCs have pleiotropic effects due to signal transduction.

Conclusion

Over more than 200 years DLCs have been applied for different purposes ranging from arrow poison to treatment of heart failure. Although the toxicity of cardiac glycoside has been considered since 1785, they are still applied in therapy due to their beneficial effects. Optimization of digoxin and digitoxin dosage based on pharmacological studies, development of assays to measure these compounds in plasma, and development of antibodies to treat digitalis toxicity have reduced the incidence of DLCs toxicity. However, they are still ranked in the top ten drugs leading to hospitalization caused by adverse drug effects. Currently, the development of new drugs and ingredients in pharmacy and food industry increase the chance of DDIs for patients that are treated with digoxin and digitoxin.

Different DLCs exist in nature and they all have the same backbone structure and mechanism of action (inhibition of Na,K-ATPase), however, they vary in their pharmacokinetic profile. Different sugar moieties and chemical substitutions at the steroid rings contribute to the different pharmacokinetic characteristics of these compounds. Currently, digoxin and digitoxin are the most prescribed DLCs for heart failure and some types of arrhythmias. In this chapter, the factors that influence DDIs with DLCs have been described. Different factors such as uptake transporters, efflux transporters, interacting drugs, and interfering physiological conditions influence the levels of DLCs and consequently their toxicity. In addition, we discussed the diversity in DLCs absorption, metabolism, excretion and the role of drug transporters at these levels. We think that knowledge about DLCs transporters will identify DLCs with less DDIs potential and better pharmacokinetic profiles.

Aim and outline of this thesis

In this thesis, research was focused on the identification and characterization of influx and efflux transporters that could play a role in the disposition of DLCs in intestine, kidney, and liver. To identify the interaction with efflux transporters, we studied a series of structurally-related DLCs for their interactions with BCRP, BSEP, and MRP1-4 in chapter 2 and with P-gp in chapter 3. Furthermore, in chapter 3 we aimed to elucidate the P-gp amino acid residues that play an important role in the interaction with DLCs.

To identify whether DLCs are substrates for ABC transporters, ATP-dependent uptake was studied in membrane vesicles overexpressing efflux transporters using an ouabain-Na,K-ATPase replacement assay and LC-MS detection methods in chapters 2 and 4, respectively. Next, an *in vivo* study was performed to confirm the *in vitro* results of a newly-identified P-gp substrate, convallatoxin. Moreover, the key amino acid residues of P-gp that play a role in convallatoxin transport were determined. The results of these studies are described in chapter 4.

In chapter 5, new P-gp substrates were identified using an accumulation assay in cells overexpressing P-gp. Additionally, two different P-gp transport assays and the criteria for the selection of a suitable transport assay to study DLCs are discussed in this chapter. In chapter 6, the interaction of DLCs with the hepatic influx transporters, Na^+-taurocholate cotransporting polypeptide (NTCP), OATP1B1, and OATP1B3 was investigated. In addition, the transport of DLCs by these transporters and new substrates of OATP1B3 were determined. Finally, the results and conclusions emanating from this thesis and their implications are discussed in chapter 7.

Chapter 2

Cardenolides Modulate Transport Activities of BSEP, BCRP and MRP1-4

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Abstract

Digitalis-like compounds (DLCs) such as digoxin and digitoxin have been applied to treat heart failure for more than 200 years. Toxicity as a consequence of drug-drug interactions at the transport level, ranked DLCs in the top ten drugs with adverse effect leading to hospitalization. Here, we investigated the interaction of cardenolide DLCs with efflux transporters; breast cancer resistance proteins (BCRP), bile salt export pump (BSEP), and multidrug resistance-associated proteins (MRPs).

The effect of 13 cardenolides on ATP-dependent uptake of [^{3}H]estrone sulphate (E_1S), [^{3}H]taurocholic acid (TCA) and [^{3}H]β-estradiol 17β-D-glucuronide ($E_2$17βG) into BCRP, BSEP and MRP1-4 overexpressing inside-out membrane vesicles, respectively, was determined. In addition, the transport of the most potent inhibitors was determined using a Na,K-ATPase-[^{3}H]ouabain replacement assay.

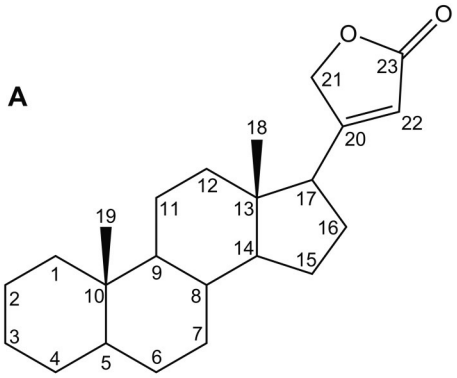
BCRP transport activity was inhibited completely by 1 mM digitoxin, digoxin, and peruvoside. This concentration of digitoxin also completely inhibited BSEP transport, whereas peruvoside and strophanthidol inhibited BSEP transport by approximately 50%. In this study strophanthidol was the strongest MRP1 and MRP3 inhibitor and digitoxigenin was the most potent MRP2 and MRP4 stimulator and inhibitor, respectively. Additionally, the present study showed that substitutions at C1, C3, C11, C12, C16 and C19 positions of the DLCs influence the interaction with BCRP, BSEP, and MRPs. Although many cardenolides interact with BCRP, BSEP, and MRP1, 2, 3, and 4 transport activities, signifying their potential role in drug-drug interactions, none of the tested compounds was a substrate for these efflux transporters in the vesicular transport assay.

Introduction

Digitalis-like compounds (DLCs), or cardiac glycosides, are classified as cardenolides with a 5-membered lactone ring (γ-lactone) or bufadienolides with a 6-membered lactone ring (δ-lactone) (Figure 1) (Barrueto *et al.*, 2006). The best-known cardenolide, digoxin, was approved by FDA for heart failure treatment in 1998 (Packer *et al.*, 1993; Uretsky *et al.*, 1993; DIG, 1997). DLCs bind and inhibit Na,K-ATPase and the resulting increased intracellular Ca^{2+} concentration stimulates heart muscle contraction (Hamlyn *et al.*, 1991; Pervaiz *et al.*, 2006). They are eliminated via renal clearance, intestinal secretion and biliary excretion (Pauli-Magnus *et al.*, 2001a). Drug-drug interactions (DDIs) of digoxin at the excretion level could occur due to the co-administration of digoxin with verapamil and quinidine leading to toxic symptoms such as headache, dizziness, fatigue, visual disturbance and nausea (Hooymans & Merkus, 1985; Belz *et al.*, 2001). Digoxin-interacting medications are substrates or inhibitors of the digoxin transporter P-glycoprotein (P-gp) (de Lannoy & Silverman, 1992). Although transport of digoxin, digitoxin, and ouabain by P-gp has been well studied (Cavet *et al.*, 1996; Brouillard *et al.*, 2001; Pauli-Magnus *et al.*, 2001a; Gozalpour *et al.*, 2014a), other major ATP-dependent efflux transporters like breast cancer resistance protein (BCRP), bile salt export pump (BSEP), and multidrug resistance associated-proteins (MRPs) could also play a role in the excretion of DLCs.

BCRP/ABCG2 is a half transporter that requires dimerization to function as an efflux transporter. It is categorized as a xenobiotic transporter that has a broad range of substrates and is expressed in placenta, prostate, small intestine, brain, colon, liver, mammary gland, and kidney (Maliepaard *et al.*, 2001; Doyle & Ross, 2003; Bart *et al.*, 2004; Fetsch *et al.*, 2006; Huls *et al.*, 2008). BSEP/ABCB11 is the major bile acid efflux transporter expressed in the canalicular membrane of hepatocytes (Strautnieks *et al.*, 1998; Byrne *et al.*, 2002; Noe *et al.*, 2002). Members of the MRP/ABCC family have broad substrate specificity from organic anions to some organic cations. Being expressed in the excretory organs such as intestine, kidney and liver, MRPs limit the accumulation of drugs in these organs (Keppler, 2011). MRP1 is highly expressed in lung, testis, kidney, skeletal and cardiac muscles, placenta, and macrophages (Flens *et al.*, 1996). Although MRP1 is located basolaterally in most tissues, it is expressed apically in endothelial cells of brain capillary (Nies *et al.*, 2004). MRP2 is apically expressed in liver, kidney, intestine, colon, bronchi, and placenta (Kruh *et al.*, 2007; Keppler, 2011). MRP3 expressing tissues include liver, gallbladder, pancreas, kidney, spleen, and adrenal cortex where it is localized basolaterally (Scheffer *et al.*, 2002). Finally, MRP4 is localized in prostate, liver, pancreas, and choroid plexus basolaterally and in kidney and brain capillary endothelial cells apically (Russel *et al.*, 2008; Keppler, 2011).

A



B

Cardenolides	1	3	5	11	12	14	16	19	Other
convallatoxin		L-rhamnose	OH			OH		O	
cymarin		D-cymarose	OH			OH		O	
digitoxigenin		OH				OH			
digitoxin		tri-D-digitoxose				OH			
digoxigenin		OH			OH	OH			
digoxin		tri-D-digitoxose			OH	OH			
dihydroouabain	OH	L-rhamnose	OH	OH		OH		OH	20-22 saturated
gitoxigenin		OH				OH	OH		
ouabagenin	OH	OH	OH	OH		OH		OH	
ouabain	OH	L-rhamnose	OH	OH		OH		OH	
peruvoside		3-O-methyl glucose				OH		O	
strophanthidin		OH	OH			OH		O	
strophanthidol		OH	OH			OH		OH	

Figure 1. Structural features of cardenolides. (A) Steroid core structure of cardenolides presenting carbon numbers, γ-lactone ring at C17. (B) Thirteen cardenolides, with different chemical substitutions on their core structure are presented.

Interaction of cardenolides with these efflux transporters has impact on the pharmacokinetics of these compounds and it might cause possible drug-drug interactions. In the present study, we have investigated the interaction of thirteen cardenolides (Figure 1) with human BCRP, BSEP, and MRP1, 2, 3, and 4 using a vesicular transport assays. Estrone sulfate (E_1S), taurocholic acid (TCA) and β-estradiol 17β-D-glucuronide ($E_2$17βG) were used as prototypic substrates of BCRP, BSEP, and MRPs, respectively. Moreover, to study the possible transport of cardenolides by these transporters, we have developed an assay using the potency of DLCs to replace Na,K-ATPase bound [^{3}H] ouabain (Flier *et al.*, 1979; Gruber *et al.*, 1980; De Pover, 1984; Kelly

et al., 1985; Halperin, 1989). Using this replacement assay, we were able to determine the uptake of cardenolides by membrane vesicles.

Materials and Methods

Materials. [6, 7-^{3}H(*N*)]Estradiol 17-β-D-glucuronide (34.3 Ci/mmol), [6, 7-^{3}H(*N*)] estrone sulfate (45 Ci/mmol), [^{3}H(G)]ouabain (25.2 Ci/mmol) and [^{3}H(G)]taurocholic acid (5 Ci/mmol) were purchased from PerkinElmer Life and Analytical Sciences (Groningen, The Netherlands). Bac-to-Bac and Gateway systems, Dulbecco's modified Eagle's medium + GlutaMAX-I culture medium, and fetal calf serum were purchased from Invitrogen (Breda, The Netherlands). Triple flasks (500 cm^2) were purchased from Sanbio BV Biological Products (Uden, The Netherlands). Estradiol 17-β-D-glucuronide ($E_2$17βG), estrone 3-sulfate potassium salt (E_1S), adenosine 5'-triphosphate magnesium salt (from bacterial source), adenosine 5'-monophosphate monohydrate (from yeast) and DLCs (convallatoxin, cymarin, digitoxigenin, digitoxin, digoxigenin, digoxin, dihydroouabain, gitoxigenin, ouabagenin, ouabain, peruvoside, strophanthidin, strophanthidol) were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Protein concentrations were determined using a Bio-Rad protein assay kit from Bio-Rad Laboratories (Veenendaal, The Netherlands). Purified swine Na,K-ATPase was kindly provided by Dr. N. U. Fedosova (Department of Biomedicine, Aarhus University, Denmark).

Generation of human BCRP, BSEP, MRP1, 2, 3, and 4 baculovirus. Full-length human BCRP, BSEP, MRP1, MRP2, MRP3, MRP4, and enhanced yellow florescent protein (eYFP) as a negative control were cloned previously (Mutsaers *et al.*, 2011; Wittgen *et al.*, 2011; Dankers *et al.*, 2012; van Beusekom *et al.*, 2013). Briefly, full length human BCRP, BSEP, MRP1, 2, 3, and 4 and eYFP cDNA, were cloned into the gateway entry vector. The gateway system was used to clone the constructs into a VSV-G-improved pFastBacDual vector with mammalian promotor (El-Sheikh *et al.*, 2008). The Bac-to-Bac system was used to produce the human BCRP, BSEP, MRP1, MRP2, MRP3, MRP4, and eYFP baculoviruses as described in the manual (Invitrogen).

Cell culture and transduction. Human embryonic kidney (HEK293) cells were grown in 10% fetal calf serum-supplemented Dulbecco's modified Eagle's medium-GlutaMAX-I at 37°C under 5% CO_2-humidified air. HEK293 cells were cultured in 500 cm^2 triple flasks. After 24 hours, culture medium was removed and 25 ml of medium and 10 ml of baculovirus (BCRP, BSEP, MRP1, MRP2, MRP3, MRP4, or eYFP) were added. Cells were incubated for 15 minutes at room temperature, after which a further 40 ml of medium and 5 mM sodium butyrate were added. Three days after transduction, cells were harvested at 3500×g for 20 minutes.

Isolation of membrane vesicles and protein analysis. Membranes were isolated according to a previously described method (El-Sheikh *et al.*, 2008). In brief, cell pellets were resuspended in ice-cold homogenizing buffer supplemented with protease inhibitors. The lysed cells were centrifuged at 100,000×g at 4°C for 30 minutes. The pellet was homogenized in ice-cold TS buffer (10 mM Tris-HEPES and 250 mM sucrose, pH 7.4) supplemented with the protease inhibitors using a tight-fitting Dounce homogenizer for 25 strokes. After centrifugation at 2000×g, 4°C for 20 min, the supernatant was centrifuged at 100,000×g at 4°C for 60 min. The membrane pellet was resuspended in ice-cold TS buffer and passed through a 27-gauge needle 25 times to form vesicles. Protein concentration was measured using the Bio-Rad protein assay kit (Bio-Rad). Crude membrane vesicles were frozen in liquid nitrogen and stored at −80°C until use.

Vesicular transport assay. A rapid filtration technique (Wittgen *et al.*, 2012) was applied to measure [^{3}H]E_1S, [^{3}H]TCA and [^{3}H]$E_2$17βG uptake into BCRP, BSEP and MRPs membrane vesicles, respectively. The 30 µl of reaction mix containing 7.5 µg prewarmed vesicles preparations, TS buffer, 10 mM $MgCl_2$, 4 mM ATP, and 0.05 µM [^{3}H]E_1S (BCRP), 0.67 µM [^{3}H]TCA (BSEP), 0.16 µM (MRP1), 0.1 µM (MRP2), 0.08 µM (MRP3) and 0.1 µM (MRP4) [^{3}H]$E_2$17βG was used. The reaction mix was supplemented with 20 µM unlabeled $E_2$17βG for MRP2 and 0.2 µM unlabeled E_1S for BCRP. After 1 minute (BCRP) or 5 minutes (BSEP and MRPs) incubation at 37°C, the reaction was stopped using 150 µl of ice-cold TS buffer. A Multiscreen$_{HTS}$ vacuum manifold filtration device (Millipore, Etten-Leur, The Netherlands) was applied to filter the diluted samples through Multiscreen$_{HTS}$-HV, 0.45-µm pore, polyvinylidene difluoride (BSEP and MRPs) and Multiscreen$_{HTS}$-HV, 0.65-µm pore, FB glass fiber (BCRP) 96-well filter plates (Millipore) that had been prewashed with TS buffer. After aspiration of the samples, the filters were washed with TS buffer twice and separated from the plate. Subsequently, 2 ml scintillation fluid was added to each sample followed by liquid scintillation counting. The uptake of [^{3}H]E_1S, [^{3}H]TCA and [^{3}H]$E_2$17βG into the membrane vesicles was determined by measuring radioactivity associated with the filters. In all experiments, ATP-dependent transport was calculated by subtracting values measured in presence of AMP from those measured in presence of ATP.

Vesicular inhibition assays. Cardenolides that inhibited the efflux transporters more than 60% were selected to evaluate their concentration-dependent effect on transporter activity. For BCRP-mediated E_1S transport, previously described assay was performed in the presence of convallatoxin, digitoxigenin, digitoxin, digoxin, and peruvoside concentrations ranging from 1 to 1000 µM. The transport of TCA by BSEP was measured in the presence of increasing concentrations of digitoxin. MRP1- and MRP3-mediated $E_2$17βG transport was also measured in the presence of strophanthidol.

Preparation of extracted membrane vesicles for Na,K-ATPase-[^{3}H]ouabain replacement assay. To determine the transport of cardenolides into membrane vesicles expressing BCRP, BSEP or MRPs, the affinity of DLCs for Na,K-ATPase was applied to develop a quantitative assay. Cardenolides (1 mM) with an inhibitory potency of more than 30% of the control were incubated with BCRP, BSEP, MRP1, MRP3, and MRP4 expressing membrane vesicles for 5 minutes at 37°C. All inhibitory cardenolides were also incubated with eYFP membrane vesicles as negative control. After aspiration of the samples, the filters were separated from the plates and transferred to Eppendorf tubes. Further, the filters were dissolved in 250 µl 98% acetonitrile + 0.1% formic acid for 45 minutes at room temperature and the filter-associated proteins were precipitated for 30 minutes at -20°C. The samples were centrifuged at 16000×g for 5 minutes and the supernatant was evaporated under N_2 gas at 37°C to reconstitute the samples. The pellets were dissolved in 50 µl distilled water containing 5% dimethyl sulfoxide (DMSO) to determine the DLC concentration in the Na,K-ATPase-[^{3}H]ouabain replacement assay (Figure 5). The above mentioned procedure was developed for P-gp expressing membrane vesicles that had been incubated for 5 minutes at 37°C with 1 mM of the P-gp substrate convallatoxin as a positive control (Gozalpour *et al.*, 2014a).

Na,K-ATPase-[^{3}H]ouabain replacement assay. To quantify the amount of cardenolides taken up into the extracted membrane vesicles, the Na,K-ATPase-[^{3}H] ouabain replacement assay was developed (De Pont *et al.*, 2009). Purified Na,K-ATPase (0.069 µg) from swine kidney (Klodos *et al.*, 2002), in sucrose buffer (250 mM sucrose, 20 mM histidine, 0.9 mM EDTA (II) pH 7.0) was added to distilled water containing 20 mM Histidine, 10 mM $MgCl_2$, 5 mM H_3PO_4, and 25 nM [^{3}H]ouabain at a final volume of 50 µl. After 2 hour incubation at room temperature, the reaction was stopped using 100 µl of ice-cold distilled water. A Multiscreen$_{HTS}$ vacuum manifold filtration device was applied to filter the diluted samples through Multiscreen$_{HTS}$-HV, 0.65-µm pore, FB glass fiber 96-well filter plate that had been prewashed with distilled water. After aspiration of the samples, the filters were washed with distilled water twice and separated from the plate. Next, 4 ml scintillation fluid was added to each sample followed by liquid scintillation counting. The binding of [^{3}H]ouabain to Na,K-ATPase was determined by measuring the radioactivity associated with the filters.

Inhibition of ouabain binding to Na,K-ATPase by cardenolides and extracted membrane vesicles. The Na,K-ATPase-[^{3}H]ouabain replacement assay was performed in the presence of convallatoxin concentrations ranging from 0 to1000 nM, cymarin, digitoxigenin, digoxigenin, digoxin, peruvoside, strophanthidin, and strophanthidol concentrations ranging from 0 to 3000 nM and of digitoxin concentrations ranging from 2000 to 3000 nM in 5% DMSO. The extracted membrane vesicles obtained from vesicular transport of cardenolides were applied in this assay to determine the amount taken up into the vesicles.

Kinetic analysis. Data analysis was done by nonlinear regression analysis using GraphPad Prism software version 5 (GraphPad Software Inc., San Diego, CA). Concentration-dependent inhibition curves were fitted to the data using the following equation: y = bottom + (top – bottom) / (1+10 $^{(\log IC50-x).\ Hill\ slope}$), in which, x and y indicate the log inhibitor concentration and uptake versus control (percentage), respectively. In all experiments, the mean value ± S.E.M of three experiments were shown. Statistical differences were tested by one-way ANOVA followed by Dunnett's *post hoc* multiple comparison and unpaired Student's t-test. A $p < 0.05$ was considered significant.

Calculation of physicochemical property of cardenolides. The octanol: water partition coefficient (shown in Table 2) was calculated using clogP add-ins in the program ChemBio3D Ultra version 12.0 (Cambridge Software).

Results

Effect of cardenolides on BCRP, BSEP, MRP1, MRP2, MRP3, and MRP4 transport activity. To study the interaction of cardenolides with BCRP and BSEP, 0.25 μM [^{3}H] E_1S and 0.67 μM [^{3}H]TCA were used, respectively. These concentrations were below the reported K_m value as determined in previous studies (Dankers *et al.*, 2012; van Beusekom *et al.*, 2013). Based on the kinetic study described previously (Wittgen *et al.*, 2011), different [^{3}H]$E_2$17βG concentrations, 0.16 μM for MRP1, 20 μM for MRP2, 0.08 μM for MRP3 and 0.10 μM for MRP4, were used to study the cardenolides-MRPs interactions.

Figures 2 and 3 show the effect of 100 and 1000 μM cardenolides on transport activity of BCRP, BSEP, MRP1, MRP2, MRP3, and MRP4. Convallatoxin, digitoxigenin, digitoxin, digoxin, peruvoside, strophanthidin, and strophanthidol inhibited BCRP-mediated E_1S transport by 50-100% at 1000 μM. Digitoxin, the most potent inhibitor of BCRP, inhibited E_1S transport by 78% at 100 μM. In addition, the BCRP-mediated E_1S transport was inhibited by 45% by the highest concentration of cymarin. Digoxigenin, dihydroouabain, and ouabagenin had stimulatory effects on E_1S transport by BCRP. Digitoxin was the single potent inhibitor of BSEP-mediated TCA transport at 1000 μM (90% inhibition). Moreover, transport activity of BSEP was inhibited by digitoxigenin, digoxigenin, and strophanthidol moderately (35-45%). BSEP-mediated TCA transport was stimulated and inhibited by 100 and 1000 μM peruvoside, respectively. In addition, convallatoxin, cymarin, and ouabagenin stimulated BSEP transport activity by 25-36%. The highest concentration of digitoxin, peruvoside, and strophanthidol inhibited MRP1 by 56%, 57%, and 73%, respectively. MRP1-mediated $E_2$17βG transport was stimulated by digitoxigenin, digoxigenin, gitoxigenin, and strophanthidin at 100 μM, whereas digoxigenin was the most potent stimulator of this transporter (43-124%). There were

no inhibitors for MRP2-mediated $E_2$17βG transport among the tested cardenolides, but cymarin, digitoxigenin, digitoxin, gitoxigenin, peruvoside and strophanthidin stimulated MRP2 transport activity more than 50%. Digitoxigenin, the most potent MRP2 stimulator, increased $E_2$17βG transport by MRP2 by 260% at 1000 μM. MRP3-mediated $E_2$17βG transport was inhibited by strophanthidin and strophanthidol at 1000 μM for 58% and 80%, respectively. Digitoxin, digoxin, and peruvoside inhibited MRP3 by 20-30% and digoxigenin was the only cardenolide that stimulated MRP3 transport (20%). Finally, MRP4-mediated $E_2$17βG transport was inhibited by 1000 μM digitoxigenin for 56%, whereas the inhibitory effect of convallatoxin, cymarin, digoxigenin, digitoxin, ouabagenin, peruvoside, and strophanthidol was less than 50%. Transport activities of BCRP, BSEP, MRP1, and MRP3 were completely inhibited in the presence of their substrates (E_1S, TCA, and $E_2$17βG), whereas MRP4 was not completely inhibited by 100 μM $E_2$17βG (74%) and MRP2 was stimulated by $E_2$17βG (55%).

Concentration-dependent inhibition of BCRP, BSEP, MRP1, and MRP3 by cardenolides. Transport activity of BCRP, BSEP, and MRPs was determined in the presence of increasing concentrations of the cardenolides that were selected as potent inhibitors (>60%) from the foregoing experiments (Figure 4).

The ATP-dependent E_1S transport activity of BCRP in the absence of cardenolides (50 ± 3 pmol/mg protein/min) was set at 100% (Figure 4) and BCRP-mediated transport in the presence of increasing concentrations of convallatoxin, digitoxigenin, digitoxin, digoxin, and peruvoside was plotted. The IC_{50} values of the most potent BCRP inhibitors, digitoxin, and digoxin were 11 and 103 μM, respectively (Figure 4). The highest concentration of digitoxigenin did not completely inhibit BCRP and convallatoxin showed the highest IC_{50} among the tested cardenolides (570 μM). Digitoxin appeared to be the only potent BSEP inhibitor. BSEP-mediated TCA transport (37 ± 4 pmol/mg protein/min) was set at 100% and was plotted against increasing digitoxin concentrations (Figure 4). Digitoxin inhibited BSEP with an IC_{50} value of 104 μM.

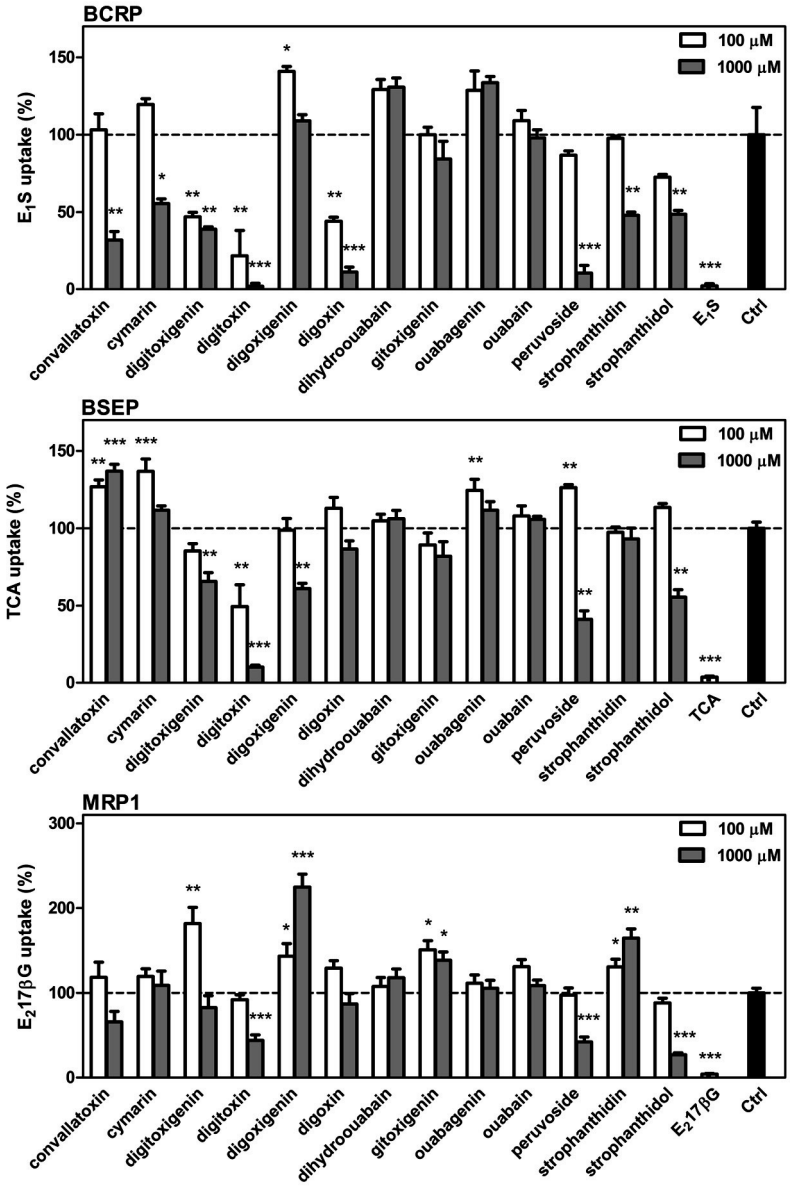


Figure 2. Inhibitory effect of thirteen cardenolides on ATP-dependent transport activity of BCRP, BSEP, and MRP1 overexpressing membrane vesicles. The transports of E_1S (for BCRP), TCA (for BSEP) and $E_2$17βG (for MRP1) were measured in the presence of 100 and 1000 μM cardenolides. ATP-dependent transport in the absence of cardenolides were 48 ± 3 for BCRP, 41 ± 8 for BSEP and 3.14 ± 0.04 for MRP1, (pmol/mg protein/min) that were set at 100% for each transporter (Ctrl) and it was also measured in the presence of 100 μM E_1S and TCA separately. Mean ± S.E.M. values of three independent experiments are shown. Each mean value was compared with Ctrl using one-way ANOVA test post-hoc Dunnett's multiple comparison, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

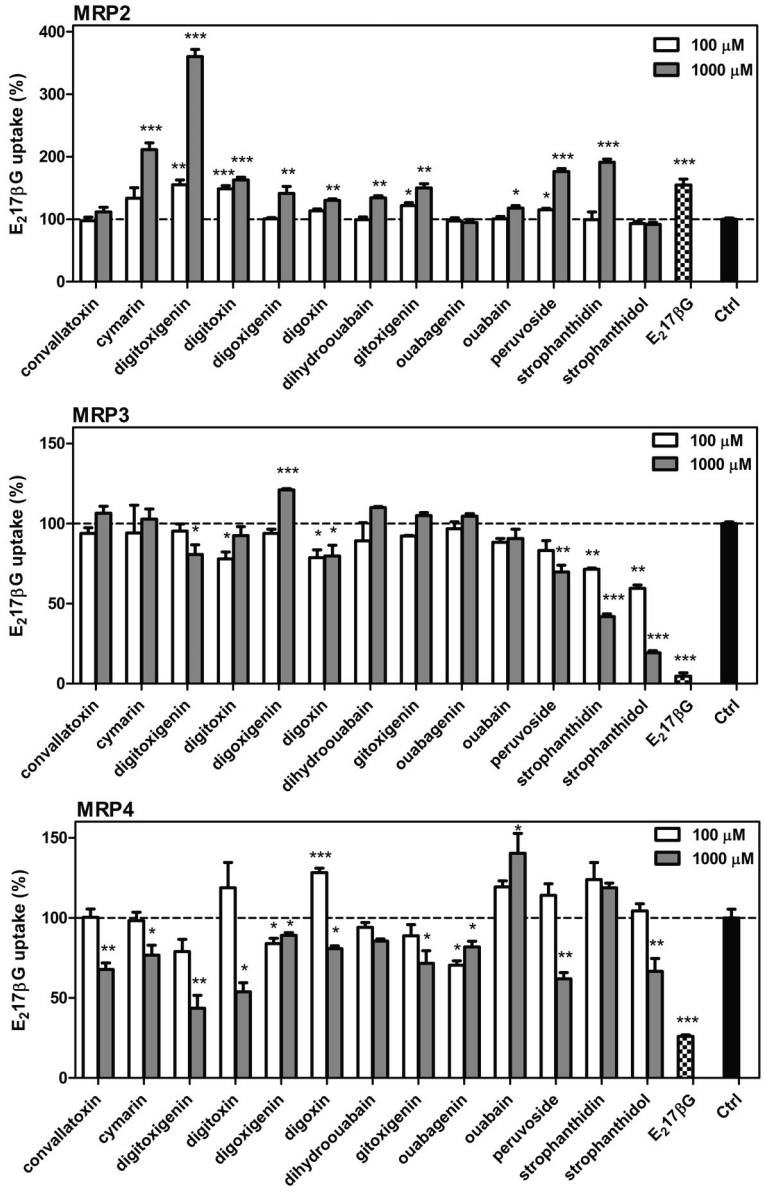


Figure 3. Inhibitory effect of thirteen cardenolides on ATP-dependent transport activity of MRP2, MRP3, and MRP4 overexpressing membrane vesicles. The transport of $E_2$17βG by MRP2, MRP3, and MRP4 were measured in the presence of 100 and 1000 μM cardenolides. ATP-dependent transport in the absence of cardenolides were 265 ± 9 for MRP2, 9.77 ± 1.35 for MRP3 and 1.737 ± 0.035 for MRP4 (pmol/mg protein/min) that were set at 100% for each transporter (Ctrl) and it was also measured in the presence of 100 μM $E_2$17βG for each transporter. Mean ± S.E.M. values of three independent experiments are shown. Each mean value was compared with Ctrl using one-way ANOVA test post-hoc Dunnett's multiple comparison, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

ATP-dependent $E_2$17βG transport in the absence of cardenolides was 3.5 ± 0.2 pmol/mg protein/min for MRP1 and 9.4 ± 0.6 pmol/mg protein/min for MRP3. These values were set at 100% and the MRP1 and MRP3 transport activities were plotted against strophanthidol concentrations. Strophanthidol inhibited MRP1 and MRP3 with IC_{50} values of 550 and 280 μM, respectively (Figure 4).

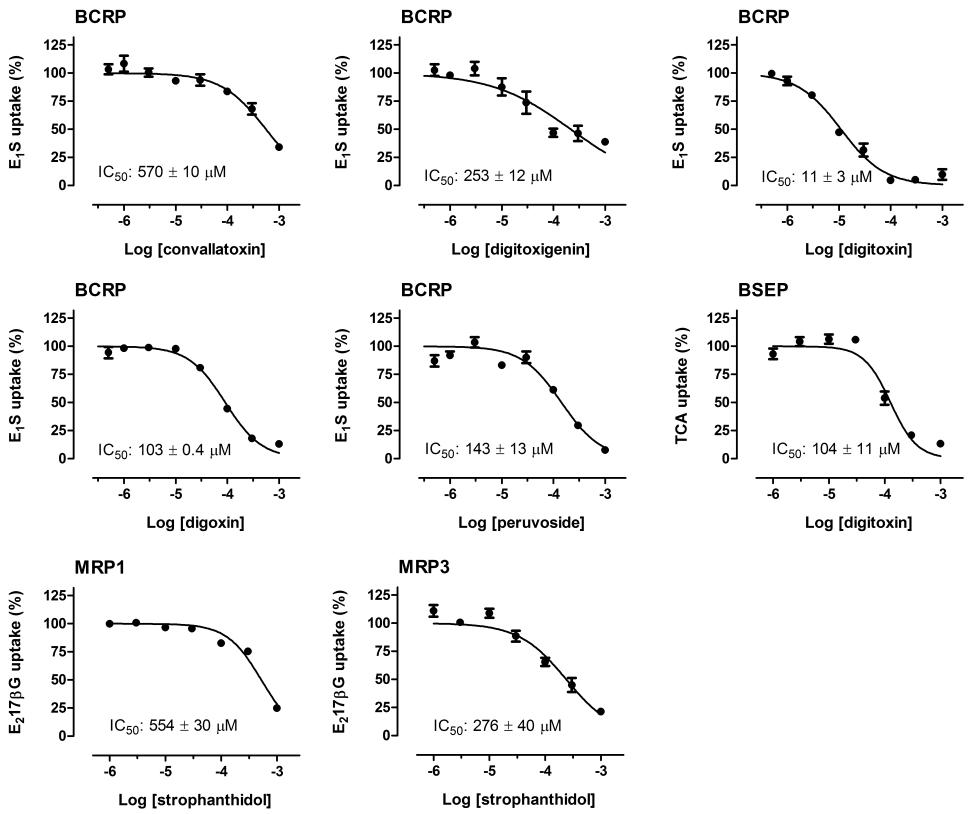


Figure 4. Concentration-dependent inhibition of BCRP, BSEP, and MRPs by cardenolides. ATP-dependent transports of E_1S by BCRP, TCA by BSEP and $E_2$17βG by MRP1 and MRP3 in the presence of increasing cardenolide concentrations were measured. Different concentrations (0.5 to 1000 μM) of convallatoxin, digitoxigenin, digitoxin, digoxin, and peruvoside were incubated with BCRP membrane vesicles in presence of 0.25 μM E_1S for 1 minute. ATP-dependent transport of 0.67 μM TCA by BSEP membrane vesicles in presence of digitoxin concentrations ranging from 1 to 1000 μM for 5 minutes was measured. MRP1 and MRP3 membrane vesicles were incubated with increasing strophanthidol concentrations from 1 to 1000 μM in presence of 0.1 μM $E_2$17βG for 5 minutes.

Cardenolide transport and Na,K-ATPase inhibition. Cardenolides that could inhibit BCRP, BSEP, MRP1, MRP3, and MRP4 by more than 30% were used to determine whether they were also substrates of these transporters. Therefore, the transporter-expressing membrane vesicles were incubated with 1000 µM cymarin (BCRP), convallatoxin (BCRP, MRP1 and 4), digitoxigenin (BCRP, BSEP, MRP4), digitoxin (BCRP, BSEP, MRP1 and 4), digoxigenin (BSEP), digoxin (BCRP), peruvoside (BCRP, BSEP, MRP1, 3, and 4), strophanthidin (BCRP, MRP3), and strophanthidol (BCRP, BSEP, MRP1, 3, and 4) in the presence of AMP or ATP at 37 °C for 5 minutes. Uptake into the membrane vesicles was quantified by the Na,K-ATPase-[^{3}H]ouabain replacement assay, based on the potency of the transported DLCs to replace ouabain (Figure 5).

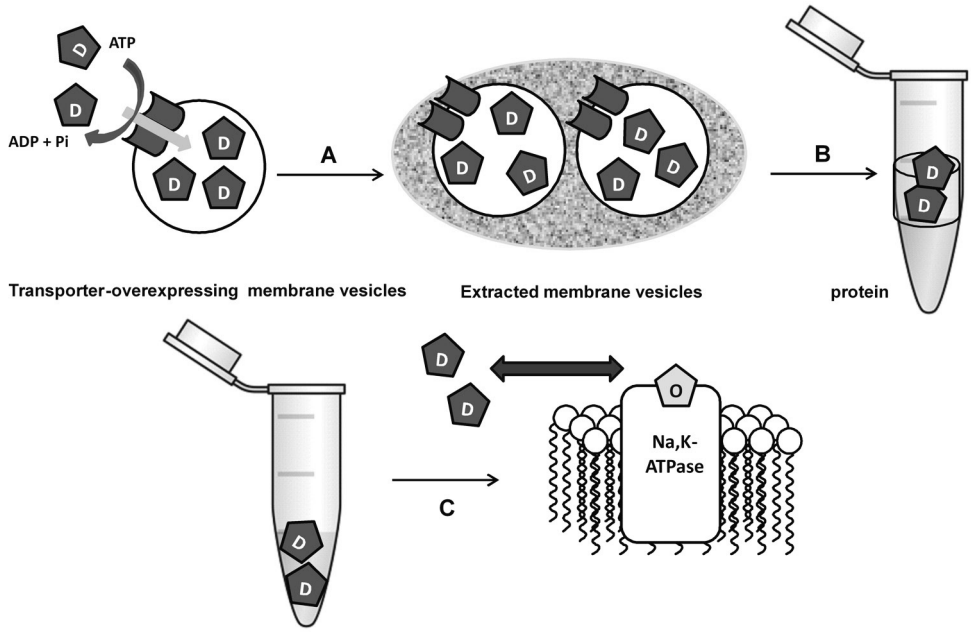


Figure 5. Extracted membrane vesicles used in the Na,K-ATPase-[^{3}H]ouabain replacement assay. Cardenolides (marked with D) were incubated with membrane vesicles expressing BCRP, BSEP, MRP1, MRP3, and MRP4 and filtered through PVDF (BSEP and MRPs) or glass fiber (BCRP) filters to remove the buffers and nonspecifically bound compound (A). The filter-associated proteins were precipitated (B) and the supernatant was analyzed in the Na,K-ATPase-[^{3}H]ouabain replacement assay (C).

As previously reported, convallatoxin is transported by P-glycoprotein membrane vesicles ATP-dependently (Gozalpour *et al.*, 2014a). The extracted membrane vesicles of P-gp and mock (eYFP) that had been incubated with 1000 µM convallatoxin or its solvent (DMSO) in the presence of AMP or ATP, were tested in the Na,K-ATPase-

[^{3}H]ouabain replacement assay (Figure 6). Ouabain binding to Na,K-ATPase in the presence of mock and extracted P-gp membrane vesicles that have been incubated with DMSO, ranged between 390-430 pmol/mg protein, which is hardly different from the binding of [^{3}H]ouabain to Na,K-ATPase in the absence of extracted membrane vesicles (Ctrl) (412 ± 9 pmol/mg protein). Na,K-ATPase ouabain binding was inhibited in the presence of mock (eYFP) and extracted P-gp membrane vesicles that were incubated with convallatoxin. Binding in the presence of extracted AMP or ATP mock-membrane vesicles was not significantly different (230 ± 20 pmol/mg protein in the presence of AMP, 210 ± 20 pmol/mg protein in the presence of ATP). The extracted P-gp-membrane vesicles that had been incubated with convallatoxin, however, showed a significant difference between the presence of ATP or AMP (41 ± 1 pmol/mg protein in the presence of ATP, 197 ± 8 pmol/mg protein in the presence of AMP) (Figure 6). We demonstrated that ATP-dependent uptake of DLCs into the membrane vesicles can be measured with the Na,K-ATPase-[^{3}H]ouabain replacement assay.

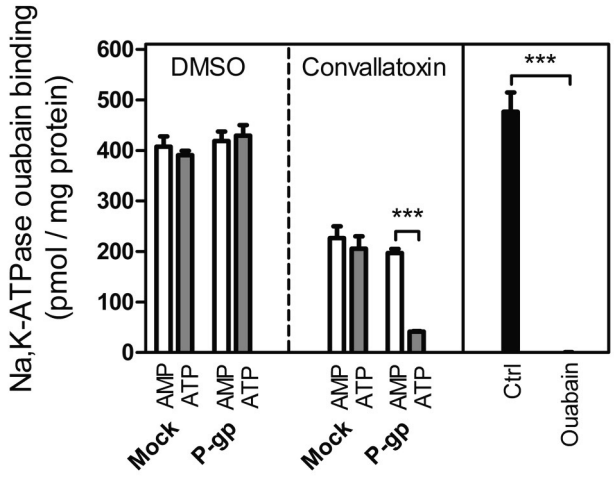


Figure 6. The effect of convallatoxin present in extracted P-gp-membrane vesicles on Na,K-ATPase-[^{3}H]ouabain replacement assay. Mock (eYFP)- and P-gp-membrane vesicles were incubated with DMSO or convallatoxin in the presence of AMP or ATP. Purified swine Na,K-ATPase (0.069 µg protein) was incubated with 25 nM [^{3}H]ouabain in the presence of extracted Mock (eYFP)- or P-gp- membrane vesicles, distilled water containing 5% DMSO (Ctrl) and 1 mM ouabain in room temperature for 2 hours. The extracted membrane vesicles that had been incubated with AMP and ATP are shown by blank and full bars, respectively.

The cardenolides, convallatoxin, cymarin, digitoxigenin, digitoxin, digoxigenin, digoxin, peruvoside, strophanthidin, and strophanthidol were studied for their transport into BCRP, BSEP and/or MRPs (except MRP2) membrane vesicles using the Na,K-ATPase-[^{3}H]ouabain replacement assay. First, the inhibitory potency of these DLCs on 25 nM ouabain binding to Na,K-ATPase was quantified. The affinity for Na,K-ATPase could be ranked as follows: strophanthidin > cymarin = convallatoxin = digitoxigenin > digoxigenin > peruvoside > digoxin > digitoxin (Table 1, Supplementary Figure 1).

Table 1. The IC_{50} values of nine cardenolides for Na,K-ATPase

	Na,K-ATPase IC_{50} (nM)
Convallatoxin	56 ± 9 [a]
Cymarin	54 ± 5
Digitoxigenin	58 ± 11
Digitoxin	2430 ± 20
Digoxigenin	80 ± 3
Digoxin	610 ± 30
Peruvoside	110 ± 20
Strophanthidin	47.3 ± 0.4
Strophanthidol	79.0 ± 0.6

[a] The IC_{50} values (Mean ± S.E.M) were obtained from three different experiments.

Binding of ouabain to Na,K-ATPase in the absence of extracted membrane vesicles was 507 ± 14 pmol/mg protein that was set at 100% (Ctrl) (Figure 7A). The extracted eYFP-membrane vesicles that had been incubated with convallatoxin, cymarin, peruvoside, strophanthidin, and strophanthidol influenced binding of ouabain to Na,K-ATPase hardly. Digitoxigenin, digitoxin, and digoxin inhibited ouabain binding to Na,K-ATPase by 95%, 85%, and 97%, respectively. Inhibition of ouabain binding by digitoxigenin, digitoxin, and digoxin suggested passive uptake into eYFP-membrane vesicles, because transport was the same in the presence of AMP or ATP (Figure 7A).

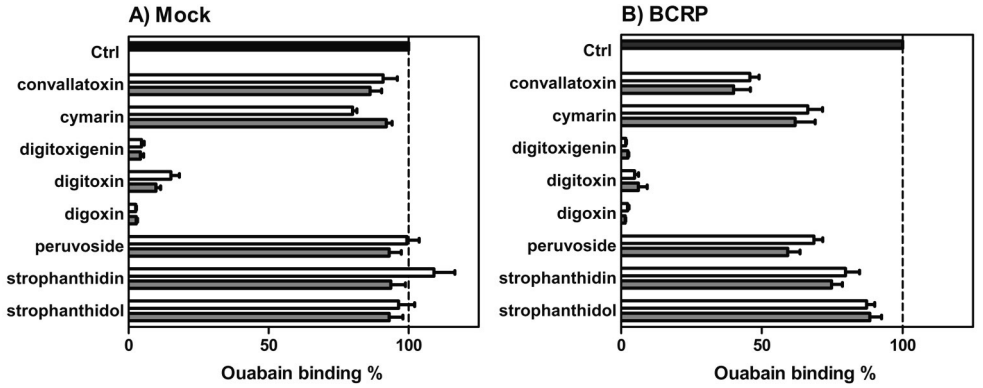


Figure 7. Inhibition of ouabain binding to Na,K-ATPase by extracted membrane vesicles. The binding of ouabain to Na,K-ATPase was measured after 2 hour incubation at room temperature in the presence of extracted Mock- (A) and BCRP-expressing membrane vesicles (B). These extracted membrane vesicles had been incubated with 1000 μM of different cardenolides in presence of AMP or ATP for 5 minutes at 37°C before performing the Na,K-ATPase-[^{3}H]ouabain replacement assay.

For BCRP the binding of ouabain to Na,K-ATPase in the absence of extracted membrane vesicles was 523 ± 11 pmol/mg protein and was set at 100% (Ctrl) (Figure 7B). The extracted membrane vesicles, which had been incubated with convallatoxin and cymarin inhibited the binding of ouabain to Na,K-ATPase, whereas the binding of ouabain was not significantly different between AMP and ATP conditions (convallatoxin: 46 ± 3% for AMP *vs*. 40 ± 6% for ATP, cymarin: 66 ± 5% for AMP *vs*. 62 ± 7% for ATP). The extracted membrane vesicles that had been incubated with digitoxigenin, digitoxin and digoxin, inhibited the [^{3}H]ouabain binding completely, however, those of peruvoside, strophanthidin and strophanthidol inhibited binding 12% to 40%. The extracted membrane vesicles of BSEP, MRP1, MRP3, and MRP4, which had been incubated with their inhibitory cardenolides, did not inhibit Na,K-ATPase ouabain binding significantly (data not shown).

Discussion

In this study, we investigated the interaction of cardenolide DLCs with the efflux transporters BCRP, BSEP, MRP1, MRP2, MRP3, and MRP4. Convallatoxin, digitoxigenin, digitoxin, digoxin, peruvoside strophanthidin, and strophanthidol inhibited BCRP-mediated E_1S transport (Figure 2). It seems that the sugar moiety at the C3 position of cardenolides improves their inhibitory effect. Digitoxin and digoxin inhibited BCRP more potently than digitoxigenin and digoxigenin, which are lacking

a sugar moiety at the C3 position (Figure 1). Additionally, convallatoxin, which has a sugar moiety at the C3 position, had a larger inhibitory effect on BCRP than strophanthidin, which contains a hydroxyl group at this position. Convallatoxin and cymarin only differ in the sugar at the C3 position. Convallatoxin seems a more potent BCRP inhibitor than cymarin. The presence of hydroxyl groups at the C12 (digoxin *vs.* digitoxin and digoxigenin *vs.* digitoxigenin) and C16 position (gitoxigenin *vs.* digitoxigenin) reduced the inhibitory effect of cardenolides for BCRP. The simultaneous presence of hydroxyl groups at positions C1 and C11 diminished the inhibitory effect (ouabagenin vs strophanthidol), whereas their presence at C5 and C19 (strophanthidol *vs*. gitoxigenin) favored BCRP inhibition. As strophanthidin and strophanthidol have the same inhibitory effect on BCRP, a carbonyl or a hydroxyl group substitution at the C19 position apparently does not influence the inhibitory effect.

Among the tested cardenolides, digitoxin, peruvoside, and strophanthidol are the most potent inhibitors of BSEP (Figure 2). By comparing the inhibition of BSEP in the presence of digitoxigenin and digitoxin, it might be concluded that the presence of sugar moiety at C3 increases the inhibitory potency of the tested cardenolides for BSEP. However, in some cases, the presence of a sugar group at C3 diminished the inhibitory effect (digoxin *vs.* digoxigenin) or improved the stimulatory effect on BSEP (convallatoxin *vs.* strophanthidin). The hydroxyl groups at C12 (digoxigenin *vs.* digitoxigenin and digoxin *vs*. digitoxin) and C16 (gitoxigenin *vs.* digitoxigenin) diminished the inhibitory effect of the cardenolides for BSEP. The hydroxyl groups at both C1 and C11 reduced the inhibitory effect (ouabagenin *vs.* strophanthidol), whereas the hydroxyl group at C19 position (strophanthidol *vs.* strophanthidin) improved it (Figure 1).

The inhibitory potency of cardenolides against MRP1 could be ranked as follows: convallatoxin < digitoxin = peruvoside < strophanthidol (Figure 2). The cardenolides with a hydroxyl group at C3 are more potent stimulators of MRP1 than those with a sugar moiety at this position (digitoxigenin *vs.* digitoxin and strophanthidin *vs.* convallatoxin). Digitoxigenin and digoxigenin with a hydroxyl group at the C3 position seemed to have a larger stimulatory potency for MRP1 compared to digoxigenin and digoxin that contain a sugar moiety at this position. Digitoxin is a stronger MRP1 inhibitor than digoxin, illustrating that the hydroxyl group at C12 does not favor MRP1 inhibition. Apparently, the hydroxyl group at the C19 position of strophanthidol improved the inhibitory effect better than the carbonyl group of strophanthidin. In addition, the combination of a hydroxyl group at C1 and C11 diminished the inhibitory effect (ouabagenin *vs.* strophanthidol), but when they are located at the C5 and C19 position, the inhibitory effect was increased (strophanthidol *vs.* gitoxigenin) (Figure 1).

There were no inhibitors of MRP2 among the cardenolides tested, however, cymarin, digitoxigenin, peruvoside, and strophanthidin stimulated E217βG transport by MRP2

(Figure 3). Digitoxigenin with a hydroxyl group at the C3 position stimulated MRP2 transport activity more potently than digitoxin, which contains a sugar moiety at this position. However, cymarin, digoxin, and ouabain, containing sugars at the C3 position, did not stimulate MRP2 more potently than non-sugar containing cardenolides, such as strophanthidin, digoxigenin, and ouabagenin. Therefore, the addition of a sugar or hydroxyl group at C3 does not result in an increased stimulatory capacity. The presence of a hydroxyl group at the C12 (digoxigenin and digoxin *vs.* digitoxigenin and digitoxin) and C16 position (gitoxigenin *vs.* digitoxigenin) reduced the stimulatory effect on MRP2. Unlike ouabain, the lactone ring at C17 in dihydroouabain is saturated, which improved the stimulatory potency of dihydroouabain for MRP2. Furthermore, the presence of a carbonyl group at C19 stimulated MRP2 more than a hydroxyl group (strophanthidin *vs.* strophanthidol) at the same position (Figure 1).

MRP3 is inhibited by strophanthidol, containing a hydroxyl group at the C19 position, more potently than strophanthidin with a carbonyl group at that position. In addition to the hydroxyl group at C19, the lack of hydroxyl groups at C1 and C11 improved the inhibitory effect of the tested cardenolides for MRP3 (ouabagenin *vs.* strophanthidol) (Figure1 and 3).

The inhibitory potency of the cardenolides on MRP4 could be ranked as follows: convallatoxin < peruvoside = strophanthidol < digitoxin < digitoxigenin (Figure 3). Based on the presence of a hydroxyl group at the C3 position in digitoxigenin and ouabagenin, which inhibited MRP4 more potently than digitoxin and ouabain, respectively, it seems that the presence of a hydroxyl group at C3 is in favor of MRP4 inhibition. However, convallatoxin with a sugar moiety at the C3 position is a more potent inhibitor as compared to strophanthidin with a hydroxyl group at this position. The hydroxyl groups at the C12 (digoxigenin and digoxin *vs.* digitoxigenin and digitoxin) and C16 positions (gitoxigenin *vs.* digitoxigenin) reduced the inhibitory effect on MRP4. Strophanthidol with a hydroxyl group at C19 position is a potent inhibitor compared with strophanthidin with a carbonyl group in this position (Figure 1).

Based on our study and comparison of the structural features of cardenolides, it can be concluded that the substitutions at the C3 position play a key role in the interactions of cardenolides with BCRP and MRP1. Interestingly, based on our observations, the simultaneous absence of hydroxyl group at C1 and C11 positions improved the inhibitory potency of cardenolides for BCRP, BSEP, MRP1, and MRP3. In addition, cardenolides lacking a hydroxyl group at position C12 were more potent inhibitors of BCRP, BSEP, MRP1, and MRP4 and more potent stimulators of MRP2. The presence of a hydroxyl group at the C19 position favored BSEP, MRP1, MRP3, and MRP4 inhibition, whereas a carbonyl group at this position was in favor of MRP2 stimulation.

Previously, we showed that the presence of a sugar moiety and hydroxyl group at the C3 and C19 positions is associated with a higher inhibitory potency against P-gp (Gozalpour *et al.*, 2013). Here, we show that substitutions of cardenolides at positions C1, C3, C11, C12, C16, and C19 play a key role in their inhibitory or stimulatory potency on BSEP, BCRP, MRP1, MRP2, MRP3, and MRP4.

In this study, we showed that digoxin inhibited BCRP transport activity, which is in line with the study of Pavek *et al.* who reported digoxin as an inhibitor of BCRP-mediated mitoxantrone transport in MDCKII-BCRP cell lines (Pavek *et al.*, 2005). Moreover, Huang *et al.* showed that the biliary and urinary excretion of digoxin is not altered in BCRP knock-out rats and digoxin is not transported by BCRP in MDCK cell line (Huang *et al.*, 2012).

It has been shown that ouabain decreases MRP1 expression reversibly in an embryonic kidney cell line and a concentration higher than 100 nM reduces MRP1-mediated carboxyfluorescein diacetate transport activity (Valente *et al.*, 2007). However, no inhibitory effect of ouabain on MRP1-mediated E217βG transport activity was observed in the present study.

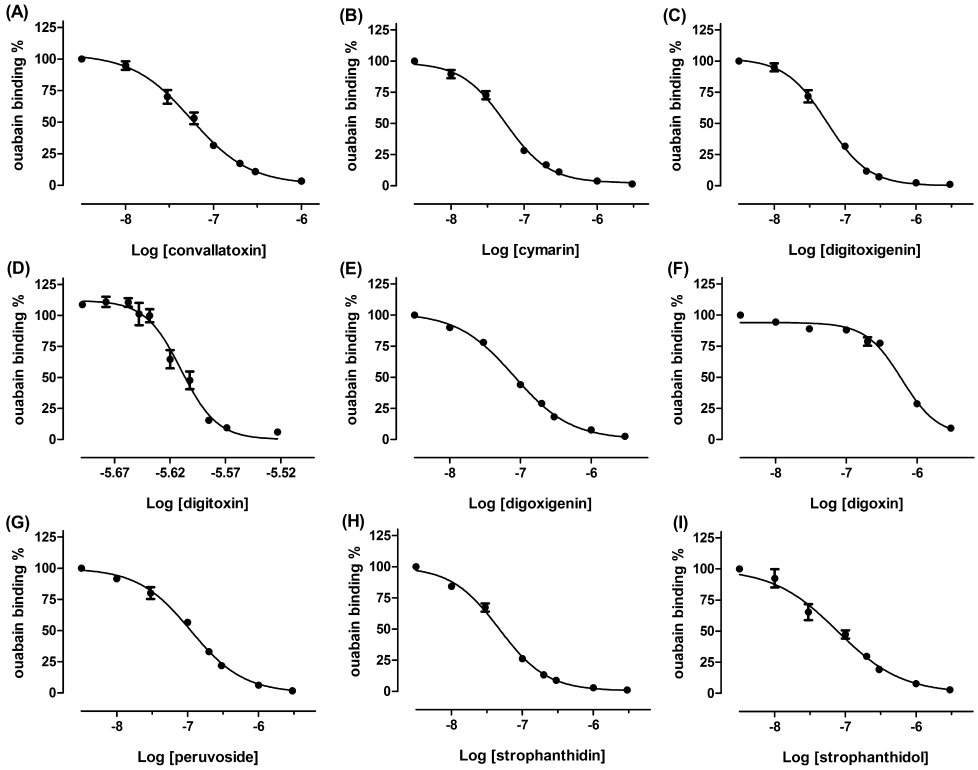
To determine whether cardenolides are transport substrates of BCRP, BSEP, and the MRPs, an indirect method based on the affinity of DLCs for Na,K-ATPase was developed. Using this assay, we were able to determine the uptake of cardenolides into membrane vesicles. Convallatoxin, cymarin, digoxigenin, peruvoside, strophanthidin, and strophanthidol did not enter the membrane vesicles in the absence or presence of ATP for all tested transporters. Interestingly, digitoxigenin, digitoxin, and digoxin accumulated into control membrane vesicles. The clogP values, that represent the lipophilicity of the compounds, were calculated for the tested cardenolides (Table 2). Digitoxigenin, digitoxin, and digoxin have the highest clogP values (2.85, 2.48, and 1.42) among tested cardenolides, which explains their passive diffusion into the membrane vesicles.

Table 2. Calculated logP values of cardenolides

Compounds	**clogP** [a]
Convallatoxin	-0.67
Cymarin	0.22
Digitoxigenin	2.48
Digitoxin	2.85
Digoxigenin	1.05
Digoxin	1.42
Dihydroouabain	-1.80
Gitoxigenin	0.32
Ouabain	-1.66
Ouabagenin	-1.32
Peruvoside	0.32
Strophanthidin	-0.30
Strophanthidol	-0.28
Proscillaridin A	2.55

[a] Calculated octanol: water partition coefficient.

In conclusion, our results show that substitutions at the C3 position influence the inhibitory potency of cardenolides for BCRP and MRP1. The hydroxyl groups at the C12 and C16 positions reduced the inhibitory potency for BCRP, BSEP, and MRP4 and the stimulatory potency for MRP2. The simultaneous presence of hydroxyl groups at C1 and C11 positions diminished the inhibitory potency for BCRP, BSEP, MRP1, and MRP3. Interestingly, the presence of a hydroxyl group at the C19 position favored BCRP, BSEP, MRP1, MRP3, and MRP4 inhibition, whereas the presence of a carbonyl group at this position favored MRP2 stimulation. In addition, the saturation of the cardenolide lactone ring seems to improve the stimulation of MRP2. Although we have shown that several DLCs interact with BCRP, BSEP, MRP1, MRP2, MRP3, and MRP4, in the vesicular assay, none of them are substrates of these transporters. The interaction of DLCs with efflux transporters can influence the distribution of other medications and might give rise to drug-drug interactions. Knowledge on DLC structure-function relationship can help to select those DLCs that have little or no interaction with efflux pumps and thereby decrease the risk of drug-drug interactions.



Supplementary Figure 1. Concentration-dependent inhibition of ouabain binding to Na,K-ATPase by cardenolides. The binding of [^{3}H]ouabain to Na,K-ATPase in the presence of increasing concentrations of convallatoxin (A) from 0 to 1000 nM, of cymarin (B), digitoxigenin (C), digoxigenin (E), digoxin (F), peruvoside (G), strophanthidin (H) and strophanthidol (I) from 0 to 3000 nM and of digitoxin (D) from 2000 to 3000 nM was plotted. The binding of [^{3}H]ouabain to Na, K-ATPase in the absence of DLCs were 469 ± 54 (A), 574 ± 34 (B), 452 ± 24.2 (C), 497± 45.7 (D), 643 ± 16 (E), 628 ± 8.6 (F), 603 ± 36.6 (G), 453 ± 17.8 (H), 581 ± 42.5 (I) pmol/mg protein (Mean ± S.E.M). These values were set at 100% and the mean value of three independent experiments was shown.

Chapter 3

Interaction of Digitalis-Like Compounds with P-glycoprotein

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Abstract

Digitalis-like compounds (DLCs), or cardiac glycosides, are produced and sequestered by certain plants and animals as a protective mechanism against herbivores or predators. Currently, the DLCs digoxin and digitoxin are used in the treatment of cardiac congestion and some types of cardiac arrhythmia, despite a very narrow therapeutic index. P-glycoprotein (P-gp; ABCB1) is the only known ATP-dependent efflux transporter that handles digoxin as a substrate.

Ten alanine mutants of human P-gp drug-binding amino acids Leu^{65}, Ile^{306}, Phe^{336}, Ile^{340}, Phe^{343}, Phe^{728}, Phe^{942}, Thr^{945}, Leu^{975}, and Val^{982} were generated and expressed in HEK293 cells with a mammalian baculovirus system. The uptake of [^{3}H]-N-methyl-quinidine (NMQ), the P-gp substrate in vesicular transport assays, was determined.

The mutations I306A, F343A, F728A, T945A, and L975A abolished NMQ transport activity of P-gp. For the other mutants, the apparent affinities for six DLCs (cymarin, digitoxin, digoxin, peruvoside, proscillaridin A, and strophanthidol) were determined. The affinities of digoxin, proscillaridin A, peruvoside and cymarin for mutants F336A and I340A were decreased two to four fold compared with wild type, whereas that of digitoxin and strophanthidol did not change. In addition, the presence of a hydroxyl group at position 12β seems to reduce the apparent affinity when the side chain of Phe336 and Phe942 is absent.

Our results showed that a δ-lactone ring and a sugar moiety at 3β of the steroid body are favorable for DLC binding to P-gp. Moreover, DLC inhibition is increased by hydroxyl groups at positions 5β and 19, whereas inhibition is decreased by those at positions 1β, 11α, 12β, and 16β. The understanding of the P-gp-DLC interaction improves our insight into DLCs toxicity and might enhance the replacement of digoxin with other DLCs that have less adverse drug effects.

Introduction

Digitalis-like compounds (DLCs), or cardiac glycosides, are made by certain plants (e.g., *Apocynaceae* family) and animals (e.g., *Bufonidae* family) (Lopez-Lazaro, 2007; Hallbook *et al.*, 2011). These toxic metabolites are produced and sequestered as a protective mechanism against herbivores or predators, and plant extracts were used as arrow poison by humans. DLCs have been employed therapeutically for the treatment of congestive heart failure for many years. Digoxin is the most prescribed DLC, and its use is characterized by a narrow therapeutic plasma concentration range (0.8–2 μg/L). Above 3 μg/L symptoms of toxicity occur, such as fatigue, nausea, vomiting, anorexia, visual disturbances, ventricular fibrillation, and ultimately death (Vivo *et al.*, 2008). Digoxin toxicity has been ranked as the second cause of drug-related hospital admissions in the United States (Abad-Santos *et al.*, 2000; Haynes *et al.*, 2009). The drug is excreted via renal clearance and intestinal secretion. Comedication with drugs such as verapamil, nifedipine, nitrendipine, propafenone, amiodarone, quinidine, cyclosporine, and itraconazole is known to increase digoxin in plasma up to toxic levels due to inhibition of transporters in the excretory organs (Belz *et al.*, 1983; Woodland *et al.*, 1998; Verschraagen *et al.*, 1999; Pauli-Magnus *et al.*, 2001a).

As digoxin interaction with P-glycoprotein (P-gp; MDR1/ ABCB1) substrates is one of the key factors in its toxicity, transport via P-gp has been extensively studied (de Lannoy & Silverman, 1992) and there is ample evidence that inhibition of P-gp transport increases the digoxin plasma levels and thereby its toxicity (Boyd *et al.*, 2000; Westphal *et al.*, 2000; Lowes *et al.*, 2003; Englund *et al.*, 2004; Shoaf *et al.*, 2011). Moreover, numerous studies have shown that P-gp polymorphisms influence digoxin plasma concentration. The synonymous polymorphisms, C3435T (Ile1145Ile) and G2677C (Ala893Pro), are the alleles that have been shown to increase orally administered digoxin levels in plasma in German population. On the other hand, there are also studies that reported that there is no association between these two polymorphisms and the digoxin plasma levels in Polish Caucasian and Japanese populations (Hoffmeyer *et al.*, 2000; Sakaeda *et al.*, 2001; Johne *et al.*, 2002; Kurzawski *et al.*, 2007). It seems that P-gp polymorphisms might influence the digoxin plasma levels population dependently. P-gp is located mainly in tissues with a barrier function like intestine (brush border membrane of enterocytes), kidney (brush border membrane of proximal tubular cells), and lumenal side of brain capillary endothelial cells (Pauli-Magnus *et al.*, 2001a; Jutabha *et al.*, 2010).

P-gp is an ATP-dependent drug pump that mediates efflux of a broad range of compounds. It is a 1280 amino acid long plasma membrane glycoprotein that consists of two halves, each of which begins with a transmembrane domain (TMD) containing six transmembrane helices (TM), followed by a nucleotide-binding domain (NBD) (Loo *et*

al., 2009). The intracellular NBDs exhibit ATPase activity that is necessary for substrate translocation, whereas the drug-binding pocket is located between the interface of two TMDs (Loo *et al.*, 2007; 2008; 2009). Recently, the crystal structure of mouse P-gp (ABCB1), which has 87% sequence identity to human P-gp, has been determined in the presence of cyclic peptide inhibitors (Aller *et al.*, 2009; Ravna *et al.*, 2009). Several amino acids involved in binding of these inhibitors were previously identified in mutagenesis studies as amino acids that play a role in substrate binding (Loo *et al.*, 2006a; 2007).

In addition to digoxin, a number of DLCs have been discovered that might also have therapeutic value in treatment of heart disease. These compounds all have the same mechanism of action as digoxin, but they have a different pharmacokinetic profile, which could be more favorable for treatment. Because P-gp activity influences digoxin plasma levels, identification of DLCs that have less interaction with P-gp could be valuable. Until now, only digoxin, digitoxin, and ouabain have been studied in P-gp transport assays, but knowledge about their P-gp-binding site is lacking (Sharom, 1995; Cavet *et al.*, 1996; Pauli-Magnus *et al.*, 2001a).

In the present study, we have expanded the knowledge about DLC interaction with human P-gp by investigating the inhibitory effect of 15 different DLCs (Figure 1) on P-gp-mediated transport of N-methyl-quinidine (NMQ) into human P-gp-containing membrane vesicles. NMQ is actively transported by P-gp in a vesicular transport assay (Hooiveld *et al.*, 2002). Our experiments showed that DLCs such as digitoxin, proscillaridin A, peruvoside, cymarin, and strophanthidol inhibited P-gp transport activity. To further understand the interaction of DLCs and NMQ with human P-gp, amino acids were mutated that could play a key role in the binding and translocation of drugs. Based on the description of drug-binding amino acids in crystallized mouse P-gp (Aller *et al.*, 2009), we selected Leu65 in TM1, Ile306 in TM5, Phe336, Ile340, and Phe343 in TM6, Phe728 in TM7, Phe942 and Thr945 in TM11, and Leu975 and val982 in TM12 of human P-gp for mutation to alanine, and thereby investigated the role of these amino acids in transport of NMQ in the presence and absence of different DLCs. Removal of the side chain resulted in loss of NMQ transport activity of five human P-gp mutants: I306A, F343A, F728A, T945A, and L975A, which seem to have key role in the transport of NMQ. However, transport activity was preserved in L65A, I306A, I340A, F942A, and V982A. These mutants were further analyzed to elucidate their role in DLC binding.

A

	substitutions at steroid positions									
cardenolides	**1β**	**3β (R1)**	**5β**	**11α**	**12β**	**14**	**16β**	**17β (R2)**	**19**	**Inhibition**
convallatoxin		L-rhamnose	OH			OH		5-member	O	++
cymarin		D-cymarose	OH			OH		5-member	O	++
digitoxigenin		OH				OH		5-member		+/-
digitoxin		tri-D-digitoxose				OH		5-member		++++
digoxigenin		OH			OH	OH		5-member		_
digoxin		tri-D-digitoxose			OH	OH		5-member		+++
dihydro-ouabain	OH	L-rhamnose	OH	OH		OH		saturated	OH	_
gitoxigenin		OH				OH	OH	5-member		_
ouabagenin	OH	OH	OH	OH		OH		5-member	OH	_
ouabain	OH	L-rhamnose	OH	OH		OH		5-member	OH	_
peruvoside		3-O-methyl glucose				OH		5-member	O	+++
strophanthidin		OH	OH			OH		5-member	O	+
strophanthidol		OH	OH			OH		5-member	OH	+++
bufadienolides										
bufalin		OH				OH		6-member		+++
proscillaridin A		L-rhamnose	4-5 double bond			OH		6-member		++++

B

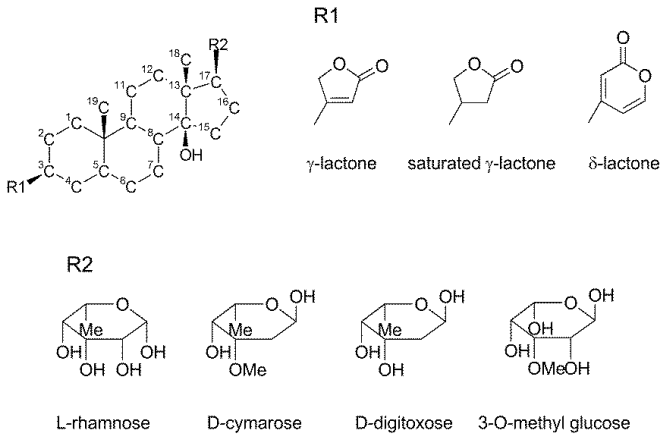

Figure 1. Structural characteristics of DLCs: cardenolides and bufadienolides. (A) DLCs have different substituents on the steroid ring, which is the core structure. (B) Steroid ring structure of DLCs illustrating carbon numbers, sugar moiety position on C-3 (R1), and lactone ring position on C-17 (R2). Based on the structure of DLCs, one of the illustrated sugars is located on C-3. The cardenolides and bufadienolides contain γ-butyrolactone (γ-lactone) and δ-valerolactone (δ-lactone) at the 17 position, respectively. γ-Lactone is saturated in dihydro forms of cardenolides such as dihydroouabain. The inhibition pattern of DLCs (1000 μM) on P-gp-mediated NMQ transport is shown as 25–50% (+), 50–75% (++), 75–90% (+++), and 90–100% (++++) inhibited transport by P-gp.

Materials and Methods

Materials. Dulbecco's Modified Eagle's Medium (DMEM) and Gluta-MAX-11 were purchased from Invitrogen (Breda, The Netherlands). [^{3}H]-NMQ (specific activity, 76.7 Ci/mmol) and unlabeled NMQ were purchased from Solvo Biotechnology (Szeged, Hungary). The Bac-to-Bac system, Cellfectin II reagent, and Grace's insect cell medium were purchased from Invitrogen. The primers for the mutagenesis were produced by Biolegio (Nijmegen, The Netherlands). The plasmid purification midiprep kit was from Genomed (Löhn, Germany). Protein concentration was measured with an assay kit from Bio-Rad Laboratories (Veenendaal, The Netherlands). DLCs (convallatoxin, cymarin, digitoxigenin, digitoxin, digoxigenin, digoxin, dihydroouabain, gitoxigenin, ouabagenin, ouabain, peruvoside, strophanthidin, strophanthidol, bufalin, and proscillaridin A) were purchased from Sigma (Zwijndrecht, The Netherlands). The mouse monoclonal antibody against P-gp, C219, and the secondary antibody, fluorescent goat anti-mouse IgG antibody IRDye 800, were purchased from Abcam (Cambridge, United Kingdom) and Rockland immunochemicals for research (Heerhugowaard, The Netherlands), respectively.

Cell culture. HEK293 cells were grown in 182-cm^2 flasks using DMEM + GlutaMAX-1 supplemented with 10% fetal calf serum at 37°C under 5% CO_2 humidified air. The cells were recultured twice a week when they were 70–80% confluent in a ratio of 1:4.

Generation of human P-gp baculovirus. Full-length human P-gp cDNA, according to Genbank accession number NM_000927, was cloned into the gateway entry clone. The gateway system was used to clone the constructs in to a VSV-G-improved pFastBacDual vector and transduce mammalian cells as described before (El-Sheikh *et al.*, 2008). The Bac-to-Bac system was used to produce human P-gp baculovirus as described in manual (Invitrogen).

Site-directed mutagenesis. A site-directed PCR was performed using pENTR-P-gp vector as template and PfuUltra II fusion HS as DNA polymerase. Ten different P-gp mutants were produced: L65A, I306A, F336A, I340A, F343A, F728A, F942A, T945A, L975A, and V982A and all mutations were confirmed by sequencing of full-length P-gp cDNA.

Transduction of HEK 293 cells with P-gp and mutant expression vectors. HEK293 cells were cultured in 182-cm^2 flasks, 13 ml of which were seeded in 500-cm^2 triple flasks (Sanbio, Uden, The Netherlands) at 85–95% confluency. After 24 h, the culture medium was removed and 25 ml of fresh medium and 10 ml of baculovirus preparations of enhanced yellow fluorescent protein (eYFP), P-gp, or P-gp mutants were added. These cells were incubated at 37°C for 20 min and finally, 40 ml of medium was added to them. Sodium butyrate was added to the cells 6 h after transduction up to a final concentration of 5 mM, and the cells were harvested 3 days later.

Isolation of membrane vesicles and protein analysis. Transduced cells were harvested by centrifugation at 3500×g for 20 min. The isolation of membrane vesicles was performed according to previously described method (El-Sheikh *et al.*, 2008). Briefly, the pellets were resuspended in ice-cold hypotonic buffer supplemented with protease inhibitors. The lysed cells were centrifuged at 100,000×g at 4°C for 30 min. The pellet was homogenized in ice-cold TS buffer (10 mM Tris-HEPES and 250 mM sucrose, pH 7.4) supplemented with the protease inhibitors using a tight-fitting Dounce homogenizer for 25 strokes. After centrifugation at 4000×g, 4°C for 20 min, the supernatant was centrifuged at 100,000×g at 4°C for 60 min. The membrane pellet was resuspended in ice-cold TS buffer and passed through a 27-gauge needle 25 times to form vesicles. Protein concentration was measured by using the Bio-Rad protein assay kit (Bio-Rad). Crude membrane vesicles were frozen in liquid nitrogen and stored at -80°C until use.

Western blot analysis. The preparations of the membrane vesicle (18 μg) were solubilized in SDS-PAGE sample buffer and separated on SDS containing 7.5% acrylamide. The protein samples were blotted on nitrocellulose membrane using iBlot dry blotting system (Invitrogen). P-gp and mutant P-gp were detected by monoclonal anti-human P-gp mouse serum antibody. β-Actin was detected by monoclonal anti-human β-actin mouse serum antibody as a loading control in eYFP and P-gp vesicle preparations. The fluorescent goat anti-mouse IgG antibody IRDye 800 was used as a secondary antibody and Odyssey infrared imaging system (Li-Cor Biosciences, Lincoln, NE) was used to visualize signals.

Vesicular transport assay. A rapid filtration technique was applied to measure [^{3}H]-NMQ uptake into membrane vesicles as described by (Wittgen *et al.*, 2011). In summary, the vesicles preparations (7.5 μg) were prewarmed at 37°C and added to TS buffer containing 10 mM $MgCl_2$, 4 mM ATP, and 0.1 μM [^{3}H]-NMQ at a final volume of 30 μl. The samples were transferred to ice to stop the reaction using 150 μl of ice-cold TS buffer. A Multiscreen$_{HTS}$ Vacuum Manifold filtration device (Millipore, Etten-Leur, The Netherlands) was applied to filter the diluted mixture through 0.65-μm pore, 96-well Multiscreen$_{HTS}$ FB (glass fiber) filters (Millipore) that were preincubated with TS buffer. After aspiration of the samples, the filters were washed with TS buffer twice. Subsequently, 2 ml scintillation fluid was added to each sample followed by liquid scintillation counting. In all experiments, net ATP-dependent transport was calculated by subtracting values measured in presence of adenosine monophosphate (AMP) from the values measured in presence of ATP. All experiments were performed in triplicates and were repeated with three different preparations of membrane vesicles.

Vesicular inhibition assays. To investigate the effect of different DLCs on P-gp-mediated NMQ transport, the previously described assay was performed in the presence of different DLCs. All DLCs were dissolved in dimethyl sulfoxide (DMSO) and

were diluted to a final concentration of 100 and 1000 μM in the incubation medium. Further, 1% DMSO (vehicle control) and 100 μM NMQ were used as the negative and positive control for inhibition, respectively. The inhibitory effect of digoxin, digitoxin, proscillaridin A, peruvoside, strophanthidol, cymarin, and bufalin on wild-type and mutant P-gp-mediated NMQ transport was investigated further by applying different concentrations of these compounds ranging from 1 to 1000 μM.

Kinetic analysis. To determine K_m and V_{max} values for P-gp-mediated NMQ transport, membrane vesicles (7.5 μg protein) were incubated with increasing concentration of NMQ in the presence of 20 nM [^{3}H]-NMQ for 1 min. The specific [^{3}H]-NMQ transport (pmol/mg protein/min) by P-gp and eYFP was determined by subtraction of AMP values from ATP values. The ATP-dependent data points were fitted by nonlinear regression analysis to the Michaelis–Menten equation. Inhibition curves were analyzed according to a one-site binding model and IC_{50} values were obtained by fitting the below equation to the data.

$$y = \text{bottom} + (\text{top} - \text{bottom}) / (1 + 10^{(\text{logIC50} - x).\ \text{Hill slope}})$$

In this equation, x and y indicate log inhibitor concentration and uptake versus control, respectively. Curve fitting was done by using GraphPad Prism software version 5 (GraphPad Software Inc., San Diego, CA). Statistical differences were tested by one-way ANOVA followed by Dunnett's *post hoc* multiple comparison. A $p < 0.05$ was considered significant.

Results

Expression of P-gp. Immunoblot analysis performed on membrane vesicles from HEK293 cells overexpressing P-gp demonstrated successful expression of P-gp at approximately 170 kDa (Figure 2A). The negative control, consisting of membrane vesicles from eYFP overexpressing HEK293 cells, showed no expression of P-gp.

Kinetics of P-gp-Mediated NMQ Transport. To determine the kinetic characteristics of P-gp in our vesicle assay, we determined the time and concentration dependency of P-gp-mediated NMQ transport. Time-dependent NMQ transport activity at 100 nM was linear up to 1 min (Figure 2B). Next, we measured concentration-dependent uptake of NMQ into membrane vesicles (Figure 2C). ATP-dependent NMQ transport reached a maximum activity (V_{max}) of 901 ± 51.3 pmol/mg protein/min (mean ± SEM). The K_m of NMQ transport via P-gp was 2.2 ± 0.48 μM, which is somewhat lower than that reported previously for P-gp expressed in Sf21 insect cells (15 μM) (Hooiveld *et al.*, 2002).

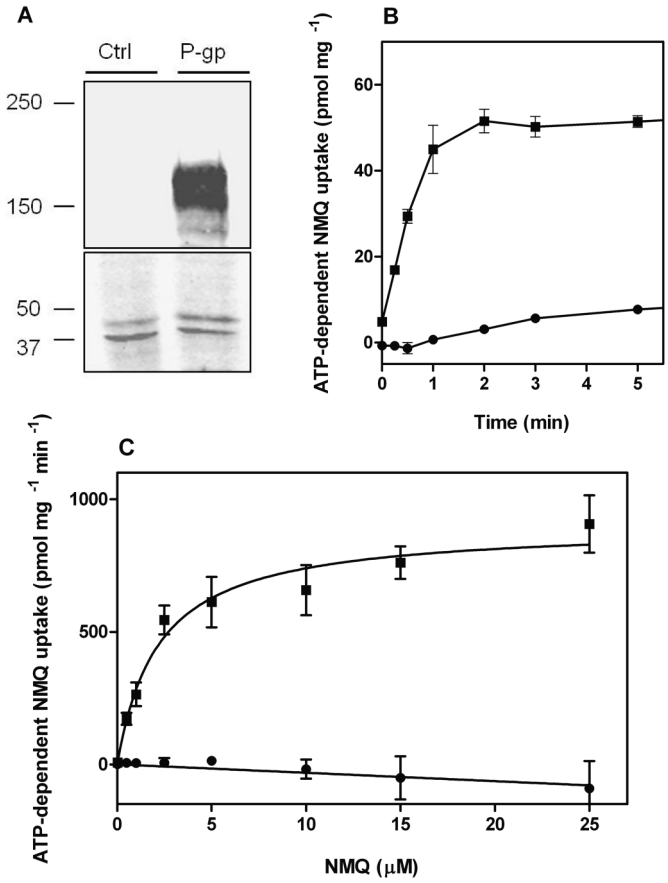

Figure 2. Immunoblot analysis of P-gp expression (A), time-dependent uptake of NMQ (B), and concentration-dependent uptake of (NMQ) (C) by membrane vesicles isolated from HEK293 cells overexpressing human P-gp (•) and eYFP (•). Top (A) represents membrane vesicles (18 μg) prepared from HEK293 cells overexpressing human P-gp and eYFP as the control. β-Actin was used as loading control for each vesicle preparation. Top (B) shows NMQ uptake of membrane vesicles (7.5 μg protein) incubated with 100nM NMQ at the indicated time points. Bottom (C) represents ATP-dependent NMQ uptake in the presence of increasing concentration of NMQ. In (B) and (C), the mean values (pmol/mg protein/min) ± SEM of three vesicle preparations are shown.

Effect of DLCs on P-gp-Mediated NMQ Transport. Two concentrations (100 and 1000 μM) of 15 DLCs were used to determine the P-gp-DLC interaction. NMQ (100 nM) transport activity of P-gp (43.7 ± 3.8 pmol/mg protein/min) was set at 100% and 100 μM unlabeled NMQ was used as a positive control (Figure 3). The large difference between the substrate concentration (100 nM) and the DLC concentration (100 and 1000 μM) ascertain that most inhibitory effects will be observed. Cymarin, digitoxin,

digoxin, peruvoside, strophanthidol, bufalin, and proscillaridin A inhibited P-gp-mediated transport of NMQ by 70–100% at 1000 μM. At 100 μM, the most potent P-gp inhibitors, digitoxin and proscillaridin A, inhibited NMQ transport by 91 and 97%, respectively. The DLC inhibitor potency of P-gp-mediated NMQ transport could be categorized as follows: digitoxin = proscillaridin A > digoxin = peruvoside = strophanthidol = bufalin > convallatoxin = digitoxigenin = cymarin > strophanthidin. Digoxigenin, dihydroouabain, gitoxigenin, ouabagenin, and ouabain did not significantly inhibit P-gp-mediated NMQ transport.

DLC Concentration-Dependent Inhibition of P-gp-Mediated NMQ Transport. The inhibitory potencies of cymarin, digitoxin, digoxin, peruvoside, proscillaridin A, and strophanthidol were quantified at 100 nM NMQ (Figure 4). The net NMQ transport by P-gp in the presence of increasing concentrations of DLCs was plotted and analyzed by nonlinear regression. The IC_{50} values of the most potent P-gp inhibitors, digitoxin and proscillaridin A, were 9 and 25 μM, respectively, whereas, cymarin, digoxin, peruvoside, and strophanthidol had 10-fold higher IC_{50} values of 432, 188, 214, and 242 μM, respectively.

Functional Expression of P-gp Mutants. To elucidate the DLC-binding site of P-gp, 10 mutants of human P-gp were constructed and expressed in HEK293 cells (Figure 5). All the indicated amino acids were replaced by alanine to remove the side chain of the residue (L65A, I306A, F336A, I340A, F343A, F728A, F942A, T945A, L975A, and V982A). First, the expression levels of wild-type and mutant enzymes were investigated by Western blot analysis (Figure 5A). Although, some variation was observed between the different batches, the average expression of the mutants was similar to that of wild type P-gp. Next, we determined the NMQ transport activity of these P-gp mutants (Figure 5B). NMQ transport activity of mutants L65A, F336A, I340A, F942A, and V982A as compared with wild-type P-gp ranged from 60 to 150%, whereas NMQ transport activity of I306A, F343A, F728A, T945A, and L975A varied between 8 and 30%.

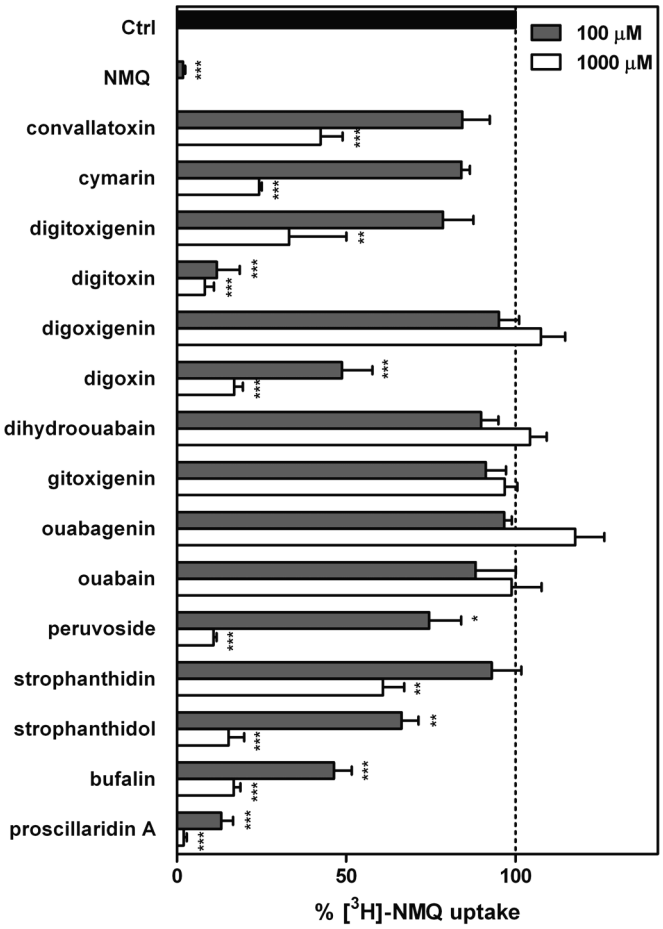

Figure 3. Inhibitory effect of different DLCs on NMQ (100 nM) transport by P-gp. Dimethyl sulfoxide (Ctrl) and cold NMQ (100 μM) were used as controls. Fifteen DLCs of 100 and 1000 μM were used in this assay. The transport activity of P-gp for 100 nM NMQ (43.7 ± 3.8 pmol/mg protein/min) in the presence of DMSO was set at 100%. Each bar represents mean ± SEM value of three experiments. Each mean value was compared with Ctrl (DMSO) using one-way ANOVA, followed by a Dunnett's *post hoc* test, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

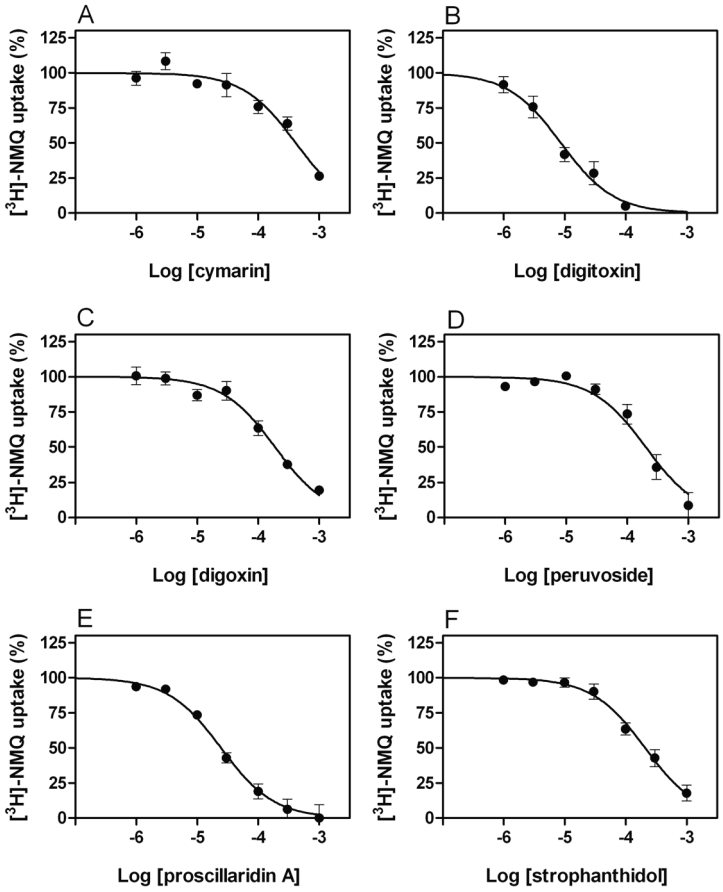

Figure 4. Inhibition of P-gp-mediated NMQ transport by DLCs. Membrane vesicles were incubated with 100 nM NMQ in the presence of cymarin (A), digitoxin (B), digoxin (C), peruvoside (D), proscillaridin A (E), and strophanthidol (F) at concentrations ranging from 0 to 1000 μM. The highest specific NMQ transport (pmol/mg protein/min) for each vesicle membrane preparation in the presence of increasing concentrations of cymarin, digitoxin, digoxin, peruvoside, proscillaridin A, and strophanthidol were 34.4 ± 3.3, 37.7 ± 3.5, 34.4 ± 3.5, 36.2 ± 6.8, 37.4 ± 7.7, and 39.2 ± 4.6 (mean ± SEM), respectively. These values were set at 100% for each vesicle membrane preparation and the percentage of mean values ± SEM of three to five preparations are shown.

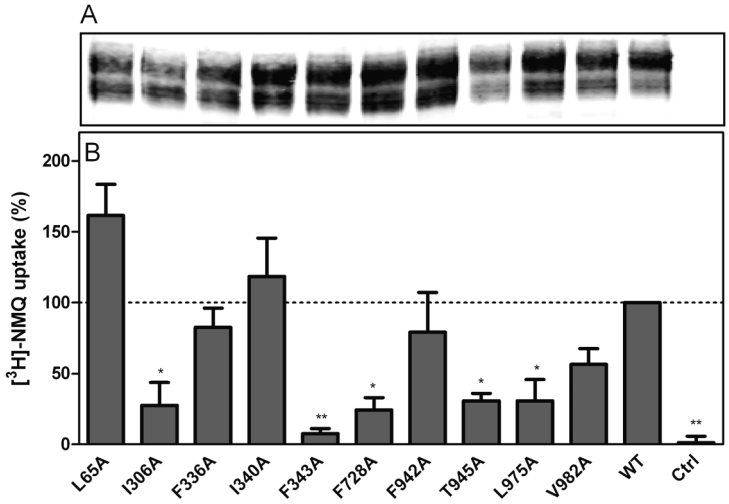

Figure 5. Western blot analysis (A) and NMQ transport activity of wild type and L65A, I306A, F336A, I340A, F343A, F728A, F942A, T945A, L975A and V982A mutant P-gp (B). Top (A) represents a Western blot analysis of membrane vesicles isolated from HEK293 cells overexpressing wild-type (WT) and mutant P-gp and eYFP as a negative control (Ctrl). In each lane, 18 µg protein was loaded. Bottom (B) represents transport activity after subtraction of AMP values from ATP values. The wild-type P-gp transport activity for NMQ (20.3 ± 4.86 pmol/mg protein/min) was set at 100%. Mean ± SEM of four different vesicle preparations are shown. The transport activity of each mutant was compared with wild-type P-gp using one-way ANOVA, followed by a Dunnett's *post hoc* test, $*p < 0.05$, $**p < 0.01$.

Concentration-Dependent Inhibition of DLCs on NMQ Transport by P-gp Mutants. The inhibitory potency of DLCs on NMQ uptake by selected P-gp mutants was analyzed. For this purpose, the DLCs that were used to determine the concentration-dependent inhibitions of P-gp-mediated NMQ transport were selected (Figure 4). In addition, five P-gp mutants (L65A, F336A, I340A, F942A, and V982A), for which NMQ transport activity was at least 50% of wild-type transport, were selected. NMQ transport of these mutants was plotted in the absence and presence of increasing concentrations of cymarin, digitoxin, digoxin, peruvoside, strophanthidol, and proscillaridin A (Figure 6). The IC_{50} value of each compound was determined for wild-type and P-gp mutants using nonlinear regression analysis (Table 1). Comparing the IC_{50} values of the mutants with those of the wild type (mutant IC_{50}/wild-type IC_{50}), L65A, and V982A showed similar values as wild type (0.6–2.2). Most remarkable, however, was the role of Phe336 and Ile340 in DLC interaction, because removal of their side chains yielded proteins that were 2.3–4.4 times less sensitive for cymarin (not significant for Ile340), digoxin (not significant), peruvoside, and proscillaridin A, whereas it hardly affected the affinity for digitoxin (1.6–2.0) or strophanthidol (0.6–1.1) (Figure 6). F942A seems to affect the digoxin binding (3.0), although not significantly.

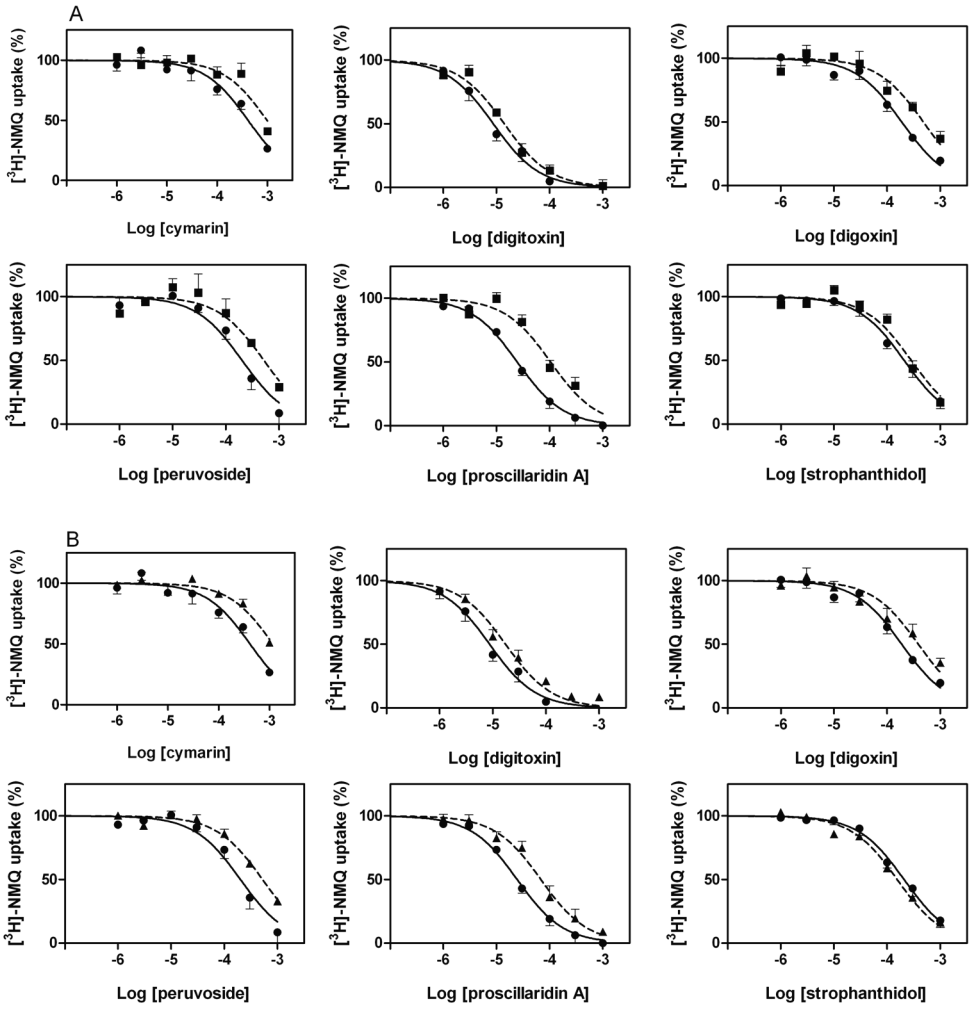

Figure 6. Concentration-dependent inhibition of wild-type and mutant P-gp-mediated [^{3}H]-NMQ transport by DLCs. Wild-type, F336A (A) and I340A (B) mutant P-gp membrane vesicles were incubated with 100 nM NMQ (containing 10 nM [^{3}H]-NMQ) in the absence or presence of indicated concentrations of cymarin, digitoxin, digoxin, peruvoside, proscillaridin A, and strophanthidol. ATP-dependent transport was determined by subtraction of uptake in presence of AMP from the value in presence of ATP. The data points have been shown by (•) for wild-type, (■) for F336A mutant and (▲) for I340A mutant P-gp. Mean ± SEM of three different vesicle preparations are shown.

Table 1. The IC_{50} values of DLCs against wild-type and mutant P-gp-mediated [^{3}H]-NMQ transport

P-gp	cymarin		digitoxin		digoxin		peruvoside		proscillaridin A		strophanthidol	
	IC_{50} (μM)	R_{IC50}	IC_{50} (μM)	R_{IC50}	IC_{50} (μM)	R_{IC50}	IC_{50} (μM)	R_{IC50}	IC_{50} (μM)	R_{IC50}	IC_{50} (μM)	R_{IC50}
Wild-type	432 ± 90		9 ± 2.1		188 ± 24		214 ± 41		25 ± 3.5		242 ± 61	
L65A	800 ± 99	1.9	17 ± 3.4	1.9	217 ± 33	1.2	469 ± 73 *	2.2	48 ± 7.2	1.9	186 ± 18	0.8
F336A	979 ± 48	2.3	14 ± 1.9	1.6	524 ± 114	2.8	528 ± 92 **	2.5	111 ± 19 **	4.4	291 ± 45	1.2
I340A	1181 ± 103 **	2.7	19 ± 4.3	2.0	439 ± 138	2.3	527 ± 37 **	2.5	79 ± 21*	3.1	156 ± 14	0.6
F942A	821 ± 256	1.9	8 ± 1.0	0.9	558 ± 145	3.0	273 ± 29	1.3	18 ± 1	0.7	187 ± 24	0.8
V982A	620 ± 106	1.4	12 ± 2.1	1.3	345 ± 73	1.8	291 ± 10	1.4	22 ± 3.4	0.9	144 ± 9	0.6

The IC_{50} values (mean ± SEM) are the means of three vesicles preparation. R_{IC50} indicates the ratio of IC_{50} for mutant to IC_{50} for the wild type. The IC_{50} values of each compound for P-gp mutant were compared with the IC_{50} values for the wild-type P-gp using one-way ANOVA, followed by a Dunnett's *post hoc* test, * $p < 0.05$, ** $p < 0.01$.

Discussion

DLCs like digoxin are used for treatment of heart failure; however, its narrow therapeutic window limits a safe application in therapy. P-gp transport is known to be a determinant of the plasma level of digoxin, and therefore drug-drug interactions with substrates or inhibitors of P-gp further complicate digoxin therapy. In this study, we investigated which structural features are important for interaction of DLCs with P-gp, and explored whether other DLCs could also interact with P-gp.

We expressed human P-gp in HEK293 cells using the mammalian baculovirus expression system described previously (El-Sheikh *et al.*, 2008; Shukla *et al.*, 2012) and determined NMQ transport into vesicles. The transport of NMQ could be inhibited by digitoxin, proscillaridin A, digoxin, peruvoside, strophanthidol, and bufalin, whereas the other DLCs did hardly (convallatoxin, cymarin, digitoxigenin, and strophanthidin) or not (digoxigenin, dihydroouabain, gitoxigenin, ouabagenin, and ouabain) inhibit this transport. Comparison of the inhibitory capacity of the different DLCs could provide information about which DLC substituent is important for binding to P-gp.

The sugar group at position 3β apparently can improve binding of most DLCs to P-gp. Digitoxin and digoxin are stronger inhibitors than digitoxigenin and digoxigenin, illustrating that the tri-D-digitoxose sugar moiety improves inhibitory potency (Figure 1). In addition, proscillaridin A, which has a L-rhamnose group at the 3β position, has a higher inhibitory potency than bufalin, which lacks a sugar group. However, addition of L-rhamnose group at 3β does not necessarily assign a good inhibitory capacity to the compound, because in the case of ouabain and ouabagenin, the sugar-containing ouabain had no inhibitory effect. From our study, it is not clear if different sugar groups contribute to the observed variation in DLC inhibitory potencies. The only difference between convallatoxin and cymarin is the sugar group (L-rhamnose vs. D-cymarose, respectively) and this apparently did not result in a different inhibitory potency.

The tested DLCs contain hydroxyl groups at positions 1β, 5β, 11α, 12β, 14, 16β, and 19. In this series, the hydroxyls at positions 1β and 11α and at positions 5β and 19 are always present together; therefore we cannot come to a conclusion on the individual contribution of hydroxyl groups at these positions (Figure 1). The presence of both hydroxyl groups at positions 1β and 11α diminishes the inhibitory potency (strophanthidol vs. ouabagenin), whereas when located at positions 5β and 19, inhibitory potency is increased (strophanthidol vs. gitoxigenin). In addition, the presence of the 12β hydroxyl group (digitoxigenin vs. digoxigenin and digitoxin vs. digoxin) and a hydroxyl group at 16β position (gitoxigenin vs. digitoxigenin) lowers the inhibitory potency. Comparing strophanthidin with strophanthidol shows that a hydroxyl at position 19 is favorable for binding over a carbonyl at this position. Comparing convallatoxin with ouabain shows

that the absence of hydroxyl groups at 1β and 11α results in a stronger inhibition than the replacement of an oxygen group by a hydroxyl at position 19. It can be concluded that all hydroxyls discussed above, lower the inhibitory potency of DLCs, except for the combination at positions 5β and 19.

In this study, we cannot state that saturation of the lactone ring influences the binding affinity significantly (ouabain vs. dihydroouabain). On the other hand, a δ-lactone ring increases inhibitory potency over a γ-lactone ring (digitoxigenin vs. bufalin).

Overall, the structure-function relationships observed indicate that a DLC with a sugar moiety at 3β, only hydroxyls at 5β, 14, and 19, and a δ-lactone ring should have a very high inhibitory capacity for P-gp. However, such a relationship might be too simple considering the polyspecific binding properties of P-gp as shown in the crystal structures of P-gp in complex with two stereoisomers of cyclic hexapeptide inhibitors (Aller *et al.*, 2009), whereas only one R stereoisomer (cyclictris-tris-(R)-valineselenazole [QZ59-RRR]) was bound, two S stereoisomers (cyclic-tris-(S)-valineselenazole [QZ59-SSS]) were bound in the binding pocket. Moreover, the location of these binding sites was not comparable.

To obtain a better indication of the precise binding site of the DLCs, we removed the side chains (mutation to Ala) of 10 amino acids. We selected these mutants from the description of *Mus musculus* P-gp (Aller *et al.*, 2009). Leu65, Ile306, Phe942, Thr945, and Leu975 are known to interact with the well-studied P-gp substrate verapamil. On the other hand, Phe336, Ile340, and Phe343 bind to the cyclic peptide inhibitor QZ59, whereas Phe728 and Val 982 interact with verapamil and QZ59. All the amino acids mutated in the present study are highly conserved between mouse and human. Leu65, Ile306, Phe336, Ile340, Phe343, Phe728, Phe942, Thr945, Leu975, and Val 982 in human correspond to Leu64, Ile302, Phe332, Ile336, Phe339, Phe724, Phe938, Thr941, Leu971, and Val 978 in *M. musculus* P-gp, respectively.

The function of these P-gp mutants was characterized by their NMQ transport activity. NMQ is a P-gp substrate that is very well transported in vesicular transport assays (Hooiveld *et al.*, 2002). It was found that P-gp mutants could be divided in two groups. The first group of mutants (I306A, F343A, F728A, T945A, and L975A) exhibited a significantly lower NMQ transport activity (8–30% of wild-type P-gp). Other studies, using site-directed mutagenesis indicated that Ile306, Phe727, Thr945, and Leu975 are also important for verapamil binding (Aller *et al.*, 2009). In addition, Phe343 was shown to play a role in binding of P-gp substrates such as vinblastine, cyclosporine A, and colchicine (Loo *et al.*, 2009). In several studies, all these amino acids have been shown to play a key role in P-gp substrate transport (Loo & Clarke, 1996; 1999b; a; Tran *et al.*, 2005; Loo *et al.*, 2006b; 2007; Lee *et al.*, 2010). The reduced transport activity we observed indicates that these residues are also involved in NMQ transport. In Figure 7,

we illustrated the position of the amino acids mutated in this study. From this figure, it appears that the amino acids that might interact with NMQ are far apart and therefore some interactions likely are indirect or in different conformations of the enzymatic cycle.

NMQ transport activity of the second group mutants (L65A, F336A, I340A, F942A, and V982A) was not significantly different from that of the wild-type P-gp (60–150%). Although Leu65, Phe942, and Val982 have been shown to play a role in verapamil transport (Loo *et al.*, 2006a; Aller *et al.*, 2009; Loo *et al.*, 2009), mutation of these amino acids did not affect NMQ transport in our assay. Other studies showed that Phe336 and Ile340, which did not influence NMQ transport significantly, seem to have a more indirect role in transport of substrates (Loo & Clarke, 2002a; b; 2005; Loo *et al.*, 2009; Ravna *et al.*, 2009) and the peptide inhibitors QZ59-RRR and QZ59-SSS bind to these amino acids in the mouse P-gp crystal structure (Aller *et al.*, 2009).

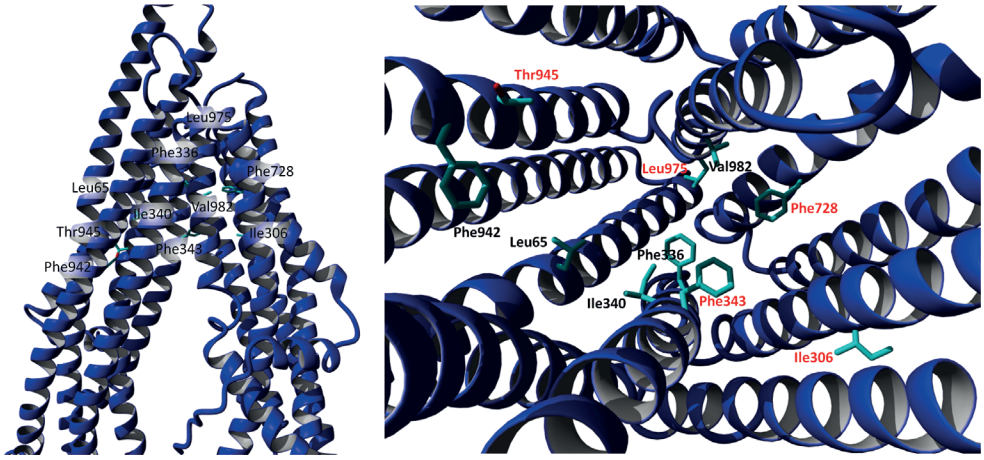

Figure 7. Position of the mutated residues in P-gp (PDB 3G5U). (A) The amino acids that are located in different P-gp TMDs have been mutated in this study. (B) The mutation of Ile306, Phe343, Phe728, Thr945, and Leu975 abolishes NMQ transport activity of P-gp that was conserved following the mutation in Leu65, Phe336, Ile340, Phe942 and Val982 (view: from cytosol to membrane).

We only investigated the effect of amino acid mutations on DLC interaction in mutants in which NMQ transport could be measured. Inhibition of NMQ transport by L65A, F942A and V982A with six DLCs showed that in only one case the mutation caused a significant difference in the IC_{50} value (ratio of 2.2) of the DLCs compared with wild-type P-gp. More remarkable were the inhibitory affinities of F336A and I340A, which were significantly different in five cases. Moreover, the IC_{50} ratio for cymarin, digoxin, peruvoside, and proscillaridin A was largely increased (IC_{50} ratio of 2.3–4.4), whereas that of digitoxin (IC_{50} ratio of 1.6–2.0) and strophanthidol (IC_{50} ratio of 0.6–

1.1) was hardly affected. It seems that the presence of a hydroxyl group at position 12β (present in digoxin and not in digitoxin) reduces the apparent affinity when the side chain of Phe336 and Phe942 is absent. Strophanthidol is the only DLC tested that has no sugar moiety (at 3β). Although the sugars (cymarose, tridigitoxose, methyl glucose, and rhamnose) have different structures, they all contain a pyranose ring and several hydroxyl groups that are sufficient for interaction with phenylalanine or isoleucine.

In conclusion, our results showed that a δ-lactone ring and a sugar moiety at 3β of the steroid body are favorable for DLC binding to P-gp. In addition, hydroxyls at 5β and 19 increase DLC inhibition, whereas those at positions 1β, 11α, 12β, and 16β decrease DLC inhibition. Although the toxicity of several DLCs is primarily due to inhibition of their therapeutic target Na,K-ATPase, interactions at the transporter level can influence their concentration at the target site. Inhibition of P-gp transport by other drugs has been shown to be an important determinant in digoxin-induced toxicity. Elucidation of the molecular mechanism of DLC translocation by P-gp will help in understanding and predicting potentially harmful drug-drug interactions and will aid in the development of DLCs that are less susceptible to pharmacokinetic interactions. We identified DLC properties that are important for their interaction with P-gp and that should be omitted in the selection of new therapeutic DLCs.

Chapter 4

Convallatoxin: A New P-glycoprotein Substrate

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Abstract

Digitalis-like compounds (DLCs), such as digoxin and digitoxin that are derived from digitalis species, are currently used to treat heart failure and atrial fibrillation, but have a narrow therapeutic index. Drug-drug interactions at the transporter level are frequent causes of DLCs toxicity. P-glycoprotein (P-gp, ABCB1) is the primary transporter of digoxin and its inhibitors influence pharmacokinetics and disposition of digoxin in the human body; however, the involvement of P-gp in the disposition of other DLCs is currently unknown.

In the present study, the transport of fourteen DLCs by human P-gp was studied using membrane vesicles originating from human embryonic kidney (HEK293) cells overexpressing P-gp. DLCs were quantified by liquid chromatography-mass spectrometry (LC-MS).

The Lily of the Valley toxin, convallatoxin, was identified as a P-gp substrate (K_m: 1.1 ± 0.2 mM) in the vesicular assay. Transport of convallatoxin by P-gp was confirmed in rat *in vivo*, in which co-administration with the P-gp inhibitor elacridar, resulted in increased concentrations in brain and kidney cortex. To address the interaction of convallatoxin with P-gp on a molecular level, the effect of nine alanine mutations was compared with the substrate N-methyl quinidine (NMQ). Phe343 appeared to be more important for transport of NMQ than convallatoxin, while Val982 was particularly relevant for convallatoxin transport.

We identified convallatoxin as a new P-gp substrate and recognized Val982 as an important amino acid involved in its transport. These results contribute to a better understanding of the interaction of DLCs with P-gp.

Introduction

Digitalis-like compounds (DLCs) are used to treat cardiac failure and atrial fibrillation because of their positive inotropic and anti-arrhythmic effects. DLCs bind and inhibit Na,K-ATPase and induce the accumulation of intracellular Na^+, which leads to reverse action of the Na^+/Ca^{2+} exchanger and subsequent increased intracellular Ca^{2+}. Subsequently, the contraction of the heart muscle is stimulated by the high concentration of Ca^{2+} inside the muscle cell (Schwinger *et al.*, 2003; Philippe & Angenot, 2005).

The foxglove derived well-known and extensively-studied DLC digoxin, has a narrow therapeutic index and above a plasma concentration of 3.84 nM (3 μg/L) toxicity symptoms occur, such as arrhythmia, anorexia, nausea, vomiting, diarrhea, abdominal pain, visual disturbances, headache, weakness and dizziness (Eichhorn & Gheorghiade, 2002; Vivo *et al.*, 2008). Drug-drug interactions at the excretion level are one of the factors playing a role in digoxin toxicity. The concomitant administration of digoxin with drugs such as amiodarone, propafenone, verapamil, quinidine, and omeprazole reduce digoxin excretion, resulting in high digoxin concentrations in plasma (Pedersen *et al.*, 1981; Belz *et al.*, 1982; Nademanee *et al.*, 1984; Marcus, 1985; Woodland *et al.*, 1997; Laer *et al.*, 1998; Fromm *et al.*, 1999; Pauli-Magnus *et al.*, 2001a; Li *et al.*, 2013). These drugs are inhibitors of the digoxin efflux transporter P-glycoprotein (P-gp) (de Lannoy & Silverman, 1992; Tanigawara *et al.*, 1992; Schinkel *et al.*, 1995).

The ATP-binding cassette transporter (ABC) family member P-gp, is the product of the Multi Drug Resistance 1 (*MDR1*, ABCB1) gene that is expressed mainly in transporting epithelia of kidney (brush border membrane of proximal tubular cells), intestine (brush border membrane of enterocytes), liver (canalicular membrane of hepatocytes) and brain capillary endothelial cells (apical membrane) (Sakaeda *et al.*, 2002; Choudhuri & Klaassen, 2006).

Although structurally different DLCs (Figure 1) have the same mechanism of action, they have different pharmacokinetic profiles (Smith, 1985). DLCs such as digoxin, methyl-digoxin, acetyl-digoxin, and digitoxin have been reported as substrates of P-gp (Pauli-Magnus *et al.*, 2001a), however, it is not known whether other DLCs are transported by P-gp as well. Identification of DLCs as substrates could provide more information about the possible drug-drug interaction at the P-gp level and could be a first step towards the development of DLC analogues that do not cause these interactions.

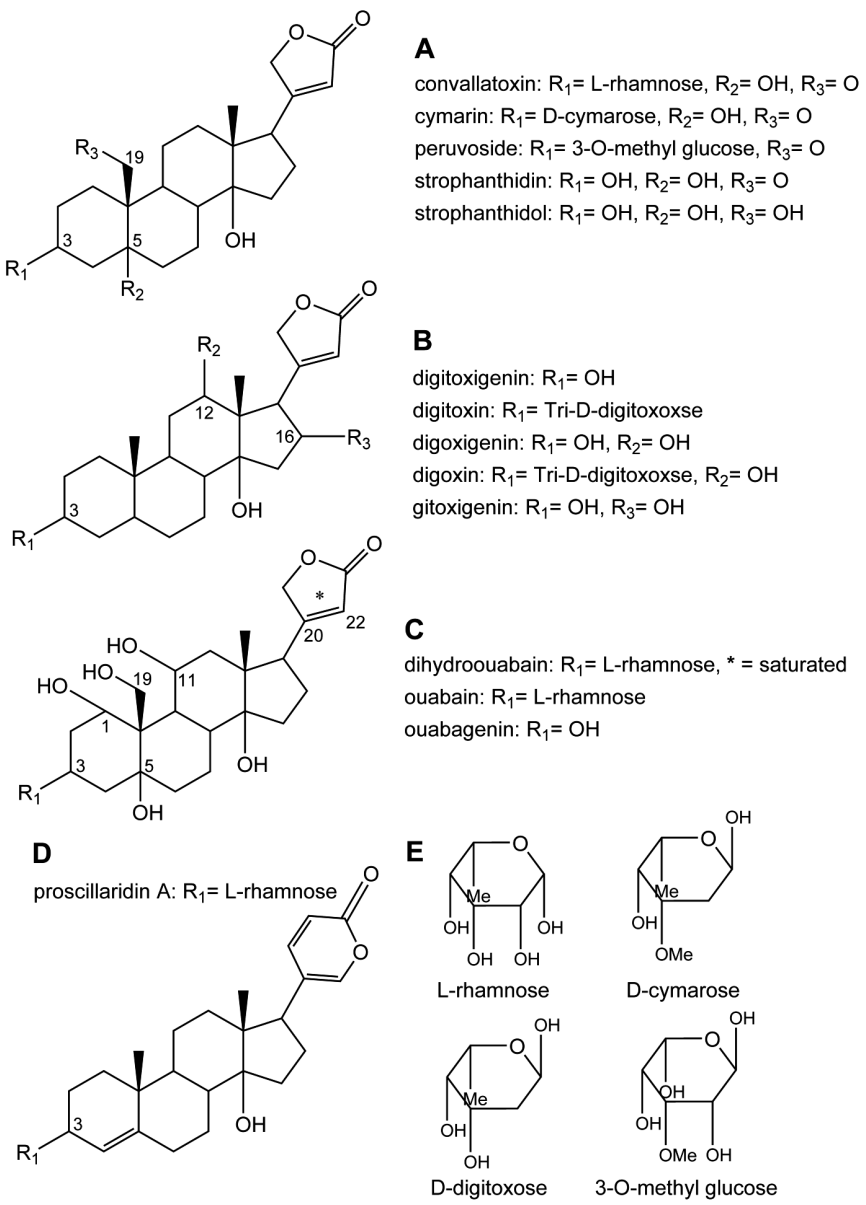

Figure1. Structure of digitalis-like compounds. Fourteen DLCs, convallatoxin, cymarin, peruvoside, strophanthidin, and strophanthidol (A), digitoxigenin, digitoxin, digoxigenin, digoxin, and gitoxigenin (B), dihydroouabain, ouabain, and ouabagenin (C) and proscillaridin A (D) are presented, categorized in four groups (A-D). DLCs are of similar structures, yet with minor differences in substitutions to the steroid core. Also note that the sugar moiety at position 3 of DLCs can contain different sugars (E) and that the lactone ring at position 17 can be 5-membered (γ-lactone) or 6-memberd (δ-lactone).

In the present study, we investigated the transport of fourteen plant derived DLCs in P-gp-overexpressing membrane vesicles using a liquid chromatography-mass spectrometry (LC-MS) method to measure DLCs directly. A rat *in vivo* study was used to confirm the findings of the vesicle transport assay. In addition, quinidine and its monoquaternary derivative, N-methyl quinidine (NMQ), were applied as the P-gp substrates to establish the functionality of our *in vivo* and *in vitro* assays, respectively. Finally, a mutagenesis study was performed to determine the important amino acids that play a role in P-gp substrate translocation.

Materials and methods

Materials. DLCs, convallatoxin (>70% purity), cymarin (>96% purity), digitoxigenin (>98% purity), digitoxin (>96% purity), digoxigenin (>98% purity), digoxin (>95% purity), dihydroouabain (>95% purity), gitoxigenin (>95% purity), ouabagenin (>95% purity), ouabain (>95% purity), peruvoside (90% purity), strophanthidin (>90% purity), strophanthidol (>96% purity), proscillaridin A (>80% purity), elacridar (GF120918), sodium butyrate, adenosine 5′-triphosphate magnesium salt (bacterial source) and adenosine 5′-monophosphate monohydrate (from yeast) were purchased from Sigma (Zwijndrecht, The Netherlands). ([^{3}H]-N-methyl quinidine ([^{3}H]-NMQ) (specific activity, 80 Ci/mmol) and unlabeled NMQ were purchased from Solvo Biotechnology (Szeged, Hungary). Dulbecco's Modified Eagle's Medium (DMEM) and Gluta-MAX-11 were purchased from Invitrogen (Breda, The Netherlands). The Bac-to-Bac system, Cellfectin II reagent, and Grace's insect cell medium were purchased from Invitrogen. The primers for the mutagenesis were produced by Biolegio (Nijmegen, The Netherlands). Protein concentration was measured with a kit from Bio-Rad Laboratories (Veenendaal, The Netherlands). The mouse monoclonal antibody against P-gp, F4, and the secondary antibody, fluorescent goat anti-mouse IgG antibody IRDye 800, were purchased from Abcam (Cambridge, United Kingdom) and Rockland immunochemicals for research (Heerhugowaard, The Netherlands), respectively. Ammonium formate (>99.0 purity) and formic acid (>98% purity) were purchased from Fluka (Steinheim, Germany). DMSO (dimethyl sulfoxide) was obtained from Merck (Darmstadt, Germany) and water was purified with a Millipore® Milli-Q system (Millipore, Bedford, MA, USA). Methanol HPLC grade was purchased from Lab Scan (Dublin, Ireland) and acetonitrile super gradient grade was obtained from VWR (Leuven, Belgium).

Cell culture. DMEM + Gluta MAX-1 supplemented with 10% fetal calf serum was used to grow HEK293 cells at 37 °C under 5% CO_2-humidified air.

Generation of baculovirus. First, full-length human P-glycoprotein (MDR1) cDNA (Genbank accession no. NM_000927) was cloned into the gateway entry clone. The P-gp construct and enhanced yellow fluorescent protein (eYFP) were cloned in to VSV-G-improved pFastBacDual vector for transduction of mammalian cells using gateway system as it was described (El-Sheikh *et al.*, 2008) previously. To produce human P-gp baculovirus, the Bac-to-Bac system was used based on manual (Invitrogen) description.

Site-directed mutagenesis. A pENTR-P-gp vector as template and PfuUltra II fusion HS as DNA polymerase were used for site-directed PCR. Nine different P-gp mutants, I306A, F336A, I340A, F343A, F728A, F942A, T945A, L975A, and V982A, were produced and sequencing of full-length P-gp cDNA was used to confirm all mutations (Gozalpour *et al.*, 2013).

Transduction of HEK293 with wild-type and mutant P-gp. HEK293 cells were cultured in 500-cm^2 triple flasks. After 24 h at 40% confluency of HEK cells, the culture medium was removed and 25 ml of fresh medium and 10 ml of baculovirus preparations of eYFP, P-gp, or P-gp mutants were added. After the incubation of these cells at 37 °C for 20 min, 40 ml of medium was added to them. 5 mM sodium butyrate was added to the cells 6 hours after transduction to stimulate protein expression by inhibition of histone deacetylase (Davie, 2003). Three days later, the cells were harvested using centrifugation at 3500×g for 10 min.

Membrane vesicle isolation and protein analysis. Membrane vesicles were isolated as described previously (El-Sheikh *et al.*, 2008). To lyse the cells the ice-cold hypotonic buffer supplemented with protease inhibitors was used to resuspend the cell pellets. After centrifugation of the lysed cells at 100,000×g at 4 °C for 30 min, the pellets were homogenized in ice-cold TS buffer (10 mM Tris-HEPES and 250 mM sucrose, pH 7.4) supplemented with the protease inhibitors using a tight-fitting Dounce homogenizer for 25 strokes. Next, the samples were centrifuged at 4000×g, 4 °C for 20 min and the consequent supernatant was centrifuged at 100,000×g at 4°C for 60 min. The ice-cold TS buffer was used to resuspend the membrane pellets and pass them through a 27-gauge needle 25 times to form vesicles. A Bio-Rad protein assay kit (Bio-Rad) was used to determine the protein concentration. After freezing in liquid nitrogen, crude membrane vesicles were stored at -80 °C until use.

Western blot analysis. Membrane vesicles (18 μg protein) were solubilized in SDS-PAGE sample buffer and separated on SDS containing 7.5% acrylamide. IBlot dry blotting system (Invitrogen) was applied to blot the protein samples on nitrocellulose membrane. Monoclonal anti-human P-gp mouse serum antibody (F4, 1: 500) followed by incubation with fluorescent goat anti-mouse IgG antibody IRDye 800 was used to detect wild type and mutant P-gp. The α-subunits of Na,K-ATPase, that were used as the loading control, were detected with the polyclonal antibody C356-M09 generated in

a rabbit (Koenderink *et al.*, 2003) followed by donkey anti-rabbit IgG antibody IRDye 680. Odyssey infrared imaging system (Li-Cor Biosciences, Lincoln, NE) was used to visualize signals.

Vesicular transport assay. The uptake of DLCs and NMQ into the membrane vesicles overexpressing P-gp was performed using a rapid filtration technique as described previously (Wittgen *et al.*, 2011). Briefly, the 30 µl reaction mix containing TS buffer, 10 mM $MgCl_2$, 4 mM AMP or ATP and 7.5 µg pre-warmed (at 37 °C) vesicles preparations was supplemented with NMQ (0.01 µM [^{3}H]-NMQ plus 0.09 µM unlabeled NMQ) or DLCs (0.1 and 1 mM). The reaction was started by incubation of the samples at 37 °C and was stopped by transferring the samples on ice and adding 150 µl of ice-cold TS buffer. The diluted samples were filtered through TS-prewashed 0.65-µm pore, 96-well Multiscreen$_{HTS}$ glass fiber filter plate using a Multiscreen$_{HTS}$-Vacuum Manifold filtration device (Millipore, Etten-Leur, The Netherlands). After washing the filters with TS buffer (0.2 ml each time) twice, 2 ml scintillation fluid was added to NMQ samples followed by liquid scintillation counting. The filter-associated radioactivity was used to determine the [^{3}H]-NMQ uptake into vesicle membrane. As DLCs samples were not radio-labeled, they were prepared to precipitate the protein for LC-MS measurement. After above-described washing steps with TS buffer, the filters were incubated with 0.2 ml mix of acetonitrile and formic acid (99.9%: 0.1%) for 30 min at room temperature and the protein was precipitated for 30 min at -20 °C. The samples were centrifuged for 5 min at 16000×g and supernatants were transferred to the new tubes for evaporation under N_2 gas at 37 °C. The totally-evaporated samples were dissolved in methanol containing 200 nM gitoxigenin as an internal standard to determine the concentration of DLCs using LC-MS. Ouabain was used as internal standard when gitoxigenin was the test compound. All experiments were performed in triplicates and were repeated with three different preparations of membrane vesicles.

Experimental Animals. For *in vivo* studies, male Wistar rats (Charles River, Kisslegg, Germany) of 240−270 g were used. The animals were permitted free access to tap water and standard lab chow. All experiments were approved by the local committee for care and use of laboratory animals and were performed according to strict governmental and international guidelines for the use of experimental animals.

Effect of P-gp on disposition of convallatoxin and quinidine in rats. Convallatoxin and quinidine with or without elacridar were dissolved in ethanol/PEG400/5% glucose solution (20: 60: 20). To study the involvement of P-gp in the disposition of convallatoxin, we investigated whether co-injection of a P-gp inhibitor would increase convallatoxin exposure of several tissues in which P-gp is known to play a role in clearance of xenobiotics from the tissue. The animals (n=12) were divided into two groups (n=6), each group was injected intravenously with convallatoxin (1.25 mg kg^{-1})

or convallatoxin plus elacridar (1.25 mg kg^{-1} + 6 mg kg^{-1}) via the tail vein. As a control experiment, to investigate whether the administered dose of elacridar was sufficient to inhibit P-gp *in vivo*, two additional groups of animals were injected intravenously with the known P-gp substrate quinidine (10 mg kg^{-1}) with or without elacridar (6 mg kg^{-1}, n=3 for each group). One hour after injection, the animals were sacrificed under isoflurane anesthesia. Urine present in the bladder was collected, as well as plasma and relevant tissues (brain, kidney, liver, and muscle). Samples were stored at -80 °C until measurement. The frozen tissues were homogenized using a Mikro-dismembrator U (B. Braun Biotech International, Melsungen) followed by addition of Hank's balanced salt solution (HBSS) supplemented with 10 mM HEPES (HBSS-HEPES), pH=7, (8 V: W). Proteins present in the samples were precipitated in two steps. First, a mix of acetonitrile with 0.1% formic acid (containing the first internal standard) was added to tissue homogenate (4: 1 V/V) and plasma and urine (8: 1 V/V). The samples were centrifuged at 2000×g for 10 min and the supernatant was transferred to the new tubes to reconstitute the samples using evaporation under N_2 gas at 37 °C. Second, the evaporated samples were dissolved in 0.2 ml methanol (containing the second internal standard) and centrifuged at 16000×g for 10 min. In the supernatant, the concentrations of quinidine and convallatoxin were determined using LC-MS, as described below. The concentrations of convallatoxin and quinidine were reported as nmol gr^{-1} tissues for the organs and nmol ml^{-1} for the plasma and the urine samples.

LC-MS quantification of DLCs. The concentrations of DLCs in the vesicular transport assays and convallatoxin and quinidine in the samples from the rat study were measured using an LC-MS system that was described previously (Gozalpour *et al.*, 2014b). For the quantitative analysis of 14 different digitalis-like compounds and quinidine, positive ion mode was used with single ion monitoring (SIM). The most abundant adducts (sodium $[M+Na]^+$ or potassium $[M+K]^+$) were used for quantification of DLCs and protonated adduct ($[M+H]^+$) was used to quantify quinidine (mass/charge ratio: 325.2 m/z).

Data analysis. To control the LC-MS system, Xcalibur® software (Thermo Scientific, San Jose, CA, USA) was used and LCquan® software (Thermo Scientific, San Jose, CA, USA) was used to analyse sample data. ATP-dependent convallatoxin transport (nmol mg $protein^{-1}$ min^{-1}) by P-gp and eYFP was determined by subtraction of AMP values from ATP values. Kinetic parameters were determined by nonlinear regression analysis of ATP-dependent transport according to the Michaelis–Menten equation. All data were expressed as mean ± S.E.M. and statistical differences were determined using an unpaired Student's t-test using GraphPad Prism software (version 5.02; Graphpad Software Inc., San Diego, CA). A $p < 0.05$ was considered significant. For the *in vivo* study, a one-sided unpaired Student's t-test was used to compare accumulated convallatoxin in the tissues of animals administered with or without elacridar.

Results

Identification of P-gp substrates in vitro. First we determined if DLCs are P-gp substrates, *in vitro*. Two concentrations (0.1 and 1 mM) of fourteen DLCs were incubated with P-gp overexpressing membrane vesicles in the absence and presence of ATP. Functional transport activity of this assay was established using NMQ as substrate of P-gp (Gozalpour *et al.*, 2013). NMQ (0.1 μM) transport by P-gp was 0.042 ± 0.003 nmol mg protein^{-1} min^{-1} in the presence of ATP compared to 0.0090 ± 0.0008 nmol mg protein^{-1} min^{-1} in the presence of AMP (normalized percentage and nmol mg protein^{-1} min^{-1} scales were shown in Figure 2A and supplementary table A, respectively).

In the presence of 0.1 mM convallatoxin we observed a significant ATP-dependent uptake of convallatoxin (0.38 ± 0.06 versus 0.062 ± 0.009 nmol mg protein^{-1} min^{-1} in the presence of ATP and AMP, respectively) and in the presence of 1 mM convallatoxin we also observed a significant ATP-dependent convallatoxin transport (2.2 ± 0.1 versus 0.42 ± 0.06 nmol mg protein^{-1} min^{-1} in the presence of ATP and AMP, respectively) (Figure 2B, supplementary table A). No significant ATP-dependent vesicular P-gp transport was observed for the other DLCs (Fig 2C-O).

Transport of convallatoxin in the absence and presence of P-gp. To confirm the transport of convallatoxin by P-gp in the membrane vesicle assay, eYFP-overexpressing membrane vesicles as an additional negative control and elacridar as the P-gp inhibitor were used (Figure 3A and B). There was no significant uptake of NMQ by eYFP-overexpressing vesicles, whereas NMQ uptake by P-gp in the presence of ATP (0.059 ± 0.002 nmol mg protein^{-1} min^{-1}) was significantly higher than when incubated with AMP (0.0070 ± 0.0002 nmol mg protein^{-1} min^{-1}) (Figure 3A). Moreover, ATP-dependent uptake was significantly decreased in the presence of 2 μM elacridar (Figure 3B). P-gp-mediated convallatoxin transport in presence of ATP (2.77 ± 0.33 nmol mg protein^{-1} min^{-1}) was significantly higher than transport in the presence of AMP (0.39 ± 0.03 nmol mg protein^{-1} min^{-1}). No significant ATP-dependent convallatoxin uptake was observed in eYFP-containing membrane vesicles (Figure 3C). In addition, ATP-dependent transport of convallatoxin was reduced significantly in the presence of elacridar (Figure 3D).

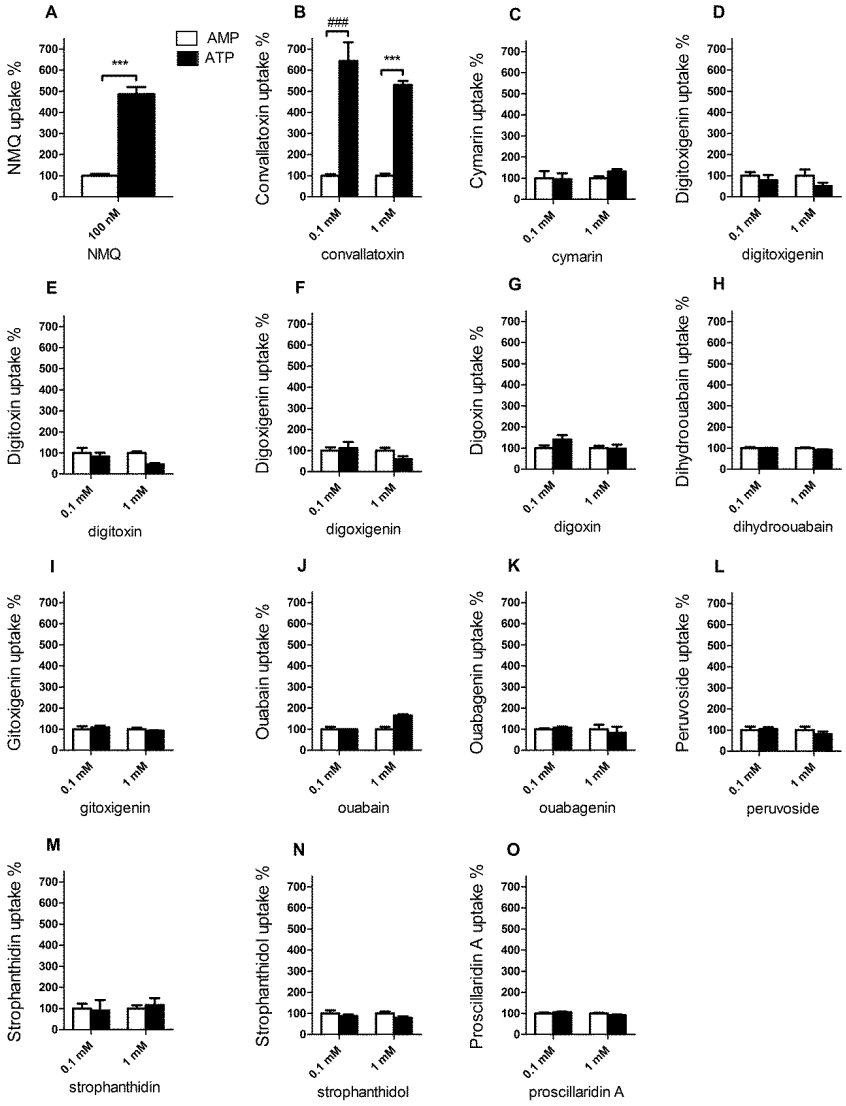

Figure 2. Uptake of DLCs in P-gp overexpressing membrane vesicles. Transport of NMQ (0.1 μM) by P-gp in the absence and presence of ATP was measured after 1 min (A). P-gp-mediated uptake of DLCs (0.1 mM and 1 mM) in membrane vesicles was measured using LC-MS. Fourteen DLCs, convallatoxin (B), cymarin (C), digitoxigenin (D), digitoxin (E), digoxigenin (F), digoxin (G), dihydroouabain (H), gitoxigenin (I), ouabain (J), ouabagenin (K), peruvoside (L), strophanthidin (M), strophanthidol (N) and proscillaridin A (O) were incubated with P-gp overexpressing membrane vesicles for 5 min in the presence of AMP or ATP. The uptake of NMQ and DLCs in the presence of AMP for each DLCs concentration was set at 100%. Each condition was performed in triplicate and mean ± S.E.M. of three independent experiments are shown. An unpaired two-sided Student's t-test was used to determine statistically significance of DLCs uptake by membrane vesicles in the absence versus presence of ATP (*** $p < 0.0001$ and ### $p = 0.0003$).

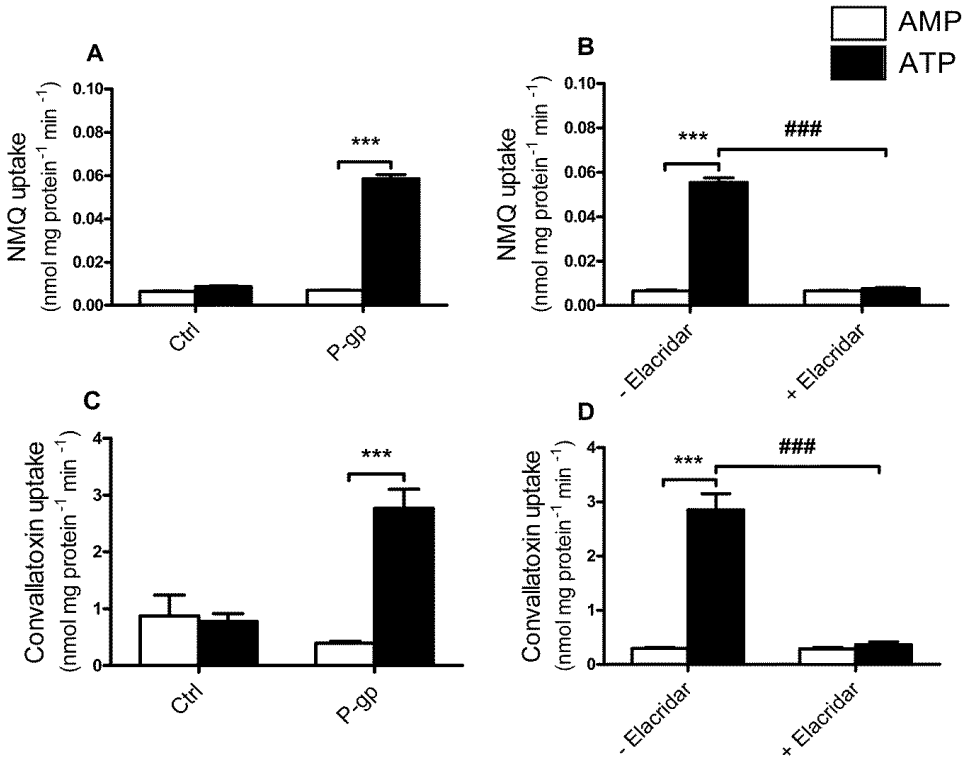

Figure 3. Uptake of NMQ and convallatoxin by membrane vesicles in the absence and presence of P-gp. Uptake of 0.1 μM NMQ and 1 mM convallatoxin by P-gp and eYFP (Ctrl) overexpressing membrane vesicles was measured in the absence and presence of AMP and ATP (A and C). P-gp-overexpressing membrane vesicles were incubated with 0.1 μM NMQ or 1 mM convallatoxin and AMP or ATP in the absence and presence of 2 μM elacridar (B and D). The incubation time for NMQ and convallatoxin experiments were 1 min and 5 min, respectively, and the mean ± S.E.M. of three independent experiments are shown. Statistically significant differences were determined using an unpaired two-sided Student's t-test: *** $p < 0.0001$ compared to the control condition and ### $p < 0.0001$ compared to the condition without elacridar.

Kinetic analysis of P-gp-mediated convallatoxin transport. To study the kinetic characteristics of convallatoxin transport by P-gp, time- and concentration-dependent uptake were measured. ATP-dependent uptake of convallatoxin was linear up to 4 min of incubation time (Figure 4). A time point of 3 min was selected to measure convallatoxin uptake at increasing concentrations. We found an apparent affinity of 1.07 ± 0.24 mM and V_{max} of 5.2 ± 0.4 nmol mg protein^{-1} min^{-1} for convallatoxin transport by P-gp (Figure 4B). Previously, we determined the kinetics of P-gp-mediated NMQ transport in the membrane vesicles (K_m: 2.2 ± 0.48 μM and V_{max}: 0.9 ± 0.05 nmol mg protein^{-1} min^{-1} (mean ± S.E.M.)) (Gozalpour *et al.*, 2013).

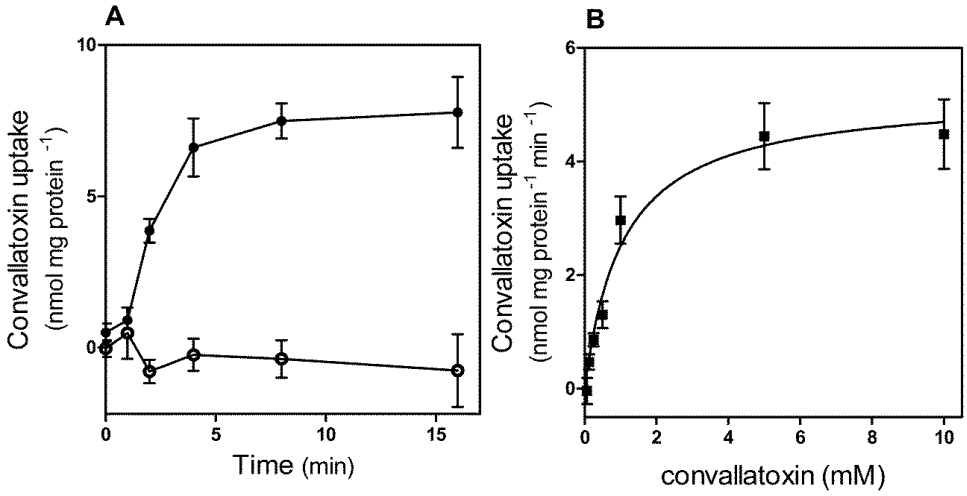

Figure 4. Kinetic characterization of P-gp-mediated convallatoxin transport in membrane vesicles. ATP-dependent convallatoxin (1 mM) uptake by eYFP (open circles) and P-gp overexpressing membrane vesicles (filled circle) was measured at the indicated time points (A). Membrane vesicles overexpressing eYFP and P-gp were incubated with increasing concentrations of convallatoxin for 3 min and ATP-dependent convallatoxin uptake is shown after subtraction of eYFP values (filled squares) (B). Mean ± S.E.M. of three independent experiments are shown.

***Disposition of convallatoxin in rats*.** The concentrations of convallatoxin and the P-gp model substrate, quinidine, were determined in different tissues, plasma, and urine 1 hour after intravenous administration of the respective compounds to rats. Both convallatoxin and quinidine were administered in the absence and presence of elacridar. Based on the study of Wittgen *et al.*, a dosage of 6 mg kg^{-1} elacridar was selected to inhibit P-gp transport activity (Wittgen *et al.*, 2012). Administration of elacridar induced a 15-fold increase in brain tissue accumulation of quinidine from 0.3 ± 0.2 nmol g^{-1} to 4.5 ± 2.4 nmol g^{-1}, indicating that the selected dose of elacridar effectively inhibited P-gp *in vivo* (Table 1).

In the absence and presence of elacridar, convallatoxin accumulated predominantly in the kidney. Moreover, the accumulation of convallatoxin in kidney cortex and brain increased when elacridar was co-administered (Table 2). Although the convallatoxin concentration was higher in the liver when co-administered with elacridar compared to administration of convallatoxin alone, this difference did not reach statistical significance. In line with the high concentrations in the kidney, convallatoxin was also present in urine, both in the presence and absence of elacridar (Table 2).

Table 1. Tissue, plasma, and urine concentrations of quinidine administered with and without elacridar.

Tissue	- Elacridar	+ Elacridar	Ratio (+ Elacridar/ - Elacridar)
Brain	0.3 ± 0.2 [a]	4.5 ± 2.4	15
Kidney	6.5 ± 1.9	8.5 ± 1.6	1.3
Liver	3.5 ± 1.5	4.0 ± 1.2	1.1
Muscle	1.4 ± 0.6	1.4 ± 0.6	1.0
Plasma	0.17 ± 0.07 [b]	0.28 ± 0.15	1.6
Urine	1.0 ± 0.4	1.3 ± 0.5	1.3

[a] nmol gr^{-1} tissue in all tissues, [b] nmol ml^{-1} in plasma or urine. Rats were dosed intravenously with quinidine (10 mg kg^{-1}) or quinidine plus elacridar (10 mg kg^{-1} plus 6 mg kg^{-1}). The concentrations of quinidine in the tissues, plasma, and urine were determined after 1 hour using LC-MS. Data are presented as mean ± S.E.M. of 3 rats for each group.

Expression and functional analysis of P-gp mutants. The important amino acids of P-gp for transport of convallatoxin were determined using a mutagenesis study. Nine amino acids were replaced by alanine (I306A, F336A, I340A, F343A, F728A, F942A, T945A, L975A, and V982A) and P-gp mutants were expressed in HEK293 cells to produce membrane vesicles. First, expression levels of the mutants were compared to wild type enzyme using western blot analysis. The Na,K-ATPase α-subunit was used as a loading control for each vesicle preparation (Figure 5A). Next, NMQ was used to determine transport activity of the mutants (Figure 5B). Wild type P-gp transported NMQ at a rate of 0.058 ± 0.005 nmol mg $protein^{-1}$ min^{-1}, which was set at 100%. NMQ transport activity of P-gp mutants I306A, F343A, and F728A was less than 40% of wild type. The transport activity of F336A, F942A, T945A, and L975A for NMQ ranged from 49% to 57%, whereas I340A showed increased activity of 120% and V982A had about the same activity as wild type (Figure 5B). A similar plot was made for the convallatoxin data, for which wild type P-gp transport activity of 2.13 ± 0.14 nmol mg $protein^{-1}$ min^{-1} was set at 100%.

Table 2. The tissues, plasma and urine concentrations of convallatoxin administered with and without elacridar

Tissue			Ratio
	- Elacridar	**+ Elacridar**	(+ Elacridar/ - Elacridar)
Brain [a]	0.5 ± 0.2	1.0 ± 0.2 [c]	2
Kidney	8.6 ± 2.2	11.9 ± 1.4	1.4
Kidney (cortex)	5.0 ± 1.0	10.8 ± 2.9 [c]	2.2
Kidney (medulla)	3.2 ± 0.7	4.1 ± 1.6	1.3
Liver	1.1 ± 0.6	2.4 ± 1.4	2.2
Muscle	0.5 ± 0.1	0.7 ± 0.3	1.4
Plasma [b]	0.26 ± 0.10	0.39 ± 0.05	1.5
Urine	4.6 ± 0.9	4.5 ± 1.0	1.0

anmol gr^{-1} tissue in all tissues, [b] nmol ml^{-1} in plasma or urine, [c] The rats were administered with convallatoxin intravenously the tail vein (1.25 mg kg^{-1}) or convallatoxin in combination with elacridar (1.25 mg kg^{-1} plus 6 mg kg^{-1}). The concentrations of convallatoxin in tissues, plasma, and urine were determined 1 hour post injection using LC-MS. Data are presented as mean ± S.E.M. of 6 rats for each group. The convallatoxin concentrations in the tissues were compared in the absence and presence of elacridar using an unpaired one-sided Student's t-test: $p < 0.05$.

Convallatoxin and NMQ transport activity were not significantly different for I306A, F336A, I340A, F728A, F942A, T945A, and L975A (Figure 5C), whereas they differed significantly for F343A and V982A (Figure 5D and E). The vesicles used for convallatoxin and NMQ transport were from the same batches, which excludes the possibility that the observed differences in transport rate are due to differences in P-gp expression. The data clearly demonstrates that transport activity of F343A for NMQ was abolished due to the mutagenesis, whereas it was partially preserved for convallatoxin. For the V982A mutant, NMQ transport was similar to that of the wild type; however, the convallatoxin transport activity was reduced two-fold.

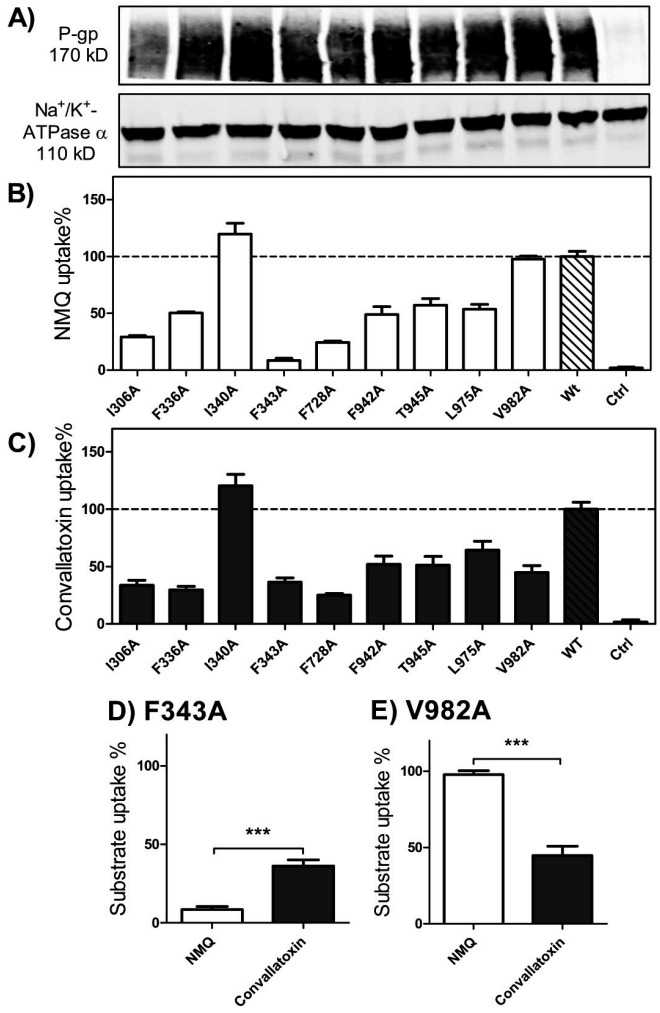

Figure 5. Western blot analysis and transport activity of P-gp mutants. Expression of wild type P-gp (WT), P-gp mutants and eYFP (Ctrl) as the negative control are shown using western blot analysis (A, top) and Na·K-ATPase was used as the loading control for each vesicle preparation (A, bottom). The ATP-dependent transport of NMQ and convallatoxin by wild-type and mutant P-gp was measured in the presence of NMQ (0.1 μM) for 1 min and 1 mM convallatoxin for 5 min (B and C). Wild-type P-gp-mediated transport of NMQ and convallatoxin was set at 100% for (B) and (C), respectively. The transport activity of F343A and V982A mutants for NMQ and convallatoxin were compared using an unpaired Student's t-test: *** $p < 0.0001$ (D and E).

Discussion

P-gp can influence the digoxin concentration in plasma. The substrates and inhibitors of P-gp interfere with the plasma concentration of digoxin and its toxicity. As all DLCs have the same therapeutic target, knowledge about their interaction with P-gp could be valuable in the development of analogues that are less susceptible to pharmacokinetic drug-drug interactions and have a larger therapeutic index.

In this study, we investigated the transport of fourteen DLCs in membrane vesicles isolated from HEK293 cells overexpressing P-gp. We showed that convallatoxin was taken up in these vesicles in an ATP-dependent fashion and that transport was inhibited by the P-gp-inhibitor elacridar. This is the first time that convallatoxin is reported as a P-gp substrate.

Convallatoxin is isolated from *Convallaria majalis* (Lily of the valley) and it inhibits Na,K-ATPase (Ozaki *et al.*, 1985; Choi *et al.*, 2006). *Convallaria majalis* extracts are used in herbal medicines to treat heart failure (Choi *et al.*, 2006). Moreover, as ornamental plant in gardens, it causes a large number of cats and dogs poisonings (Cortinovis & Caloni, 2013). Human poisoning by exposure to this plant can occur in the case of accidental leaves ingestion or the incorrect use of herbal medicine (Alexandre *et al.*, 2012). Although it was shown that convallatoxin uptake by rat intestine is transporter-mediated, there is no information about the efflux transporters involved in the pharmacokinetics of this compound (Lauterbach, 1968). It was shown in Caco-2 cells that convallatoxin, unlike digitoxin and bufalin, hardly inhibited digoxin secretion (Cavet *et al.*, 1996), which can be explained by the low affinity we observed in this study (approximately 1 mM).

As the purity of convallatoxin used in this study was more than 70%, it could be speculated that the remaining impurities influence the results. Previously, we showed that DLCs such as digitoxin, digoxin, peruvoside, proscillaridin A and strophanthidol could inhibit P-gp-mediated NMQ transport (Gozalpour *et al.*, 2013). NMQ is a monoquaternary derivative of the P-gp substrate quinidine (Kusuhara *et al.*, 1997; Fromm *et al.*, 1999; Hooiveld *et al.*, 2002) that has a very high P-gp affinity (2.2 μM) compared to convallatoxin (1 mM). In our previous study, convallatoxin did not affect NMQ transport (Gozalpour *et al.*, 2013), therefore, convallatoxin and its impurities do no inhibit P-gp. Moreover, convallatoxin impurities (30%) do not influence our conclusion that convallatoxin is a P-gp substrate, as this is based on a direct measurement of convallatoxin. However, we cannot exclude that it could influence our quantitative estimation of convallatoxin affinity (K_m) for P-gp.

Interestingly, in this study digoxin, as the prototypical P-gp substrate, was not transported by P-gp in HEK membrane vesicles. The vesicular transport assay is not

suitable to study the transport of hydrophobic compounds like digoxin. In contrast to hydrophilic compounds like convallatoxin, hydrophobic compounds will diffuse extensively through the lipid membrane of the vesicle (von Richter *et al.*, 2009; Szeremy *et al.*, 2011). In our study, digoxin was highly accumulated in the membrane vesicles and its P-gp-mediated transport was concealed. The fact that digoxin is lipophilic and the vesicles are not suitable to study its transport is reflected in the lack of other studies using the P-gp membrane vesicles for digoxin transport. In most studies, P-gp-mediated digoxin transport has been studied in transwell transport assays with polarized cells that contain uptake and efflux transporters (Pauli-Magnus *et al.*, 2001a; Balimane *et al.*, 2004; Balimane & Chong, 2005). Hydrophobicity of the other DLCs tested here could be categorized based on their calculated octanol: water partition coefficient (clogP) (Gozalpour *et al.*, 2014b) as follows: digitoxin (clogP: 2.85) > proscillaridin A (2.55) > digitoxigenin (2.48) > gitoxigenin (2.25) > digoxin (1.42) > digoxigenin (1.05) > peruvoside (0.32) > cymarin (0.22) > strophanthidol (- 0.28) > strophanthidin (- 0.30) > convallatoxin (- 0.67) > ouabagenin (- 1.32) > ouabain (- 1.66) > dihydroouabain (- 1.80), which indicates why convallatoxin transport could be well measured in the vesicular system in contrast to digoxin.

Our results pointing to the involvement of P-gp in the disposition of convallatoxin obtained in P-gp-overexpressing membrane vesicles were in line with the results obtained in the *in vivo* rat study. Although convallatoxin was previously used to determine the lethal dosage in rats (Lorenz & Stoeckert, 1958), its pharmacokinetics in rats have not been studied before nor have mechanistic investigations been conducted that studied P-gp as a determinant of convallatoxin disposition. The high accumulation of convallatoxin in rat kidney and its presence in urine illustrate that renal excretion is the main elimination route of convallatoxin. Moreover, a significantly higher accumulation of convallatoxin in kidney cortex was found when convallatoxin was co-administered with elacridar, whereas kidney medulla exposure was not affected. This underlines the involvement of P-gp, as this transporter is expressed predominantly in renal proximal tubule cells situated in the kidney cortex and not in the medulla (Cordon-Cardo *et al.*, 1990). The large difference between the accumulation of quinidine and convallatoxin in rat tissues might reflect the lower affinity of convallatoxin for P-gp compared with quinidine. In contrast to human P-gp, there are two genes, mdr1a, and mdr1b, for P-gp in rodents. Mdr1a (Mdr3) is expressed in intestine, liver, brain and testis, whereas Mdr1b (Mdr1) is expressed in adrenal glands, placenta, ovaries, and uterus (Schinkel *et al.*, 1994; Schinkel *et al.*, 1995). In this study, the high accumulation of quinidine in brain in the presence of elacridar, confirms the role of Mdr1a in quinidine disposition that was reported previously (Kusuhara *et al.*, 1997; Fromm *et al.*, 1999). As both Mdr1a and Mdr1b are expressed in the kidney of rodents, we could not determine if Mdr1a or Mdr1b was responsible for convallatoxin disposition in rat kidney.

It should be noted however, that the increased accumulation of convallatoxin in kidney cortex and brain, and the tendency towards a higher increase of convallatoxin in liver could also be related to an effect of elacridar on convallatoxin efflux transporters other than P-gp. Elacridar is also known to inhibit BCRP (Cooray *et al.*, 2002; Matsson *et al.*, 2009), however, only limited information is available on DLC handling by BCRP. Digoxin has been found as BCRP inhibitor by Pavek *et al.*, but it is not a substrate (Pavek *et al.*, 2005). From pilot experiments from our lab there are also no indications that convallatoxin is a BCRP substrate, nor has it been reported as such in literature. The possibility of the involvement of BCRP in convallatoxin disposition nevertheless remains open and has to be further investigated. With regard to the liver, it could be hypothesized that the relatively limited (and non-significant) increase of convallatoxin concentrations in the presence of elacridar may be due to a dual inhibition of uptake and efflux systems. In this respect, we previously, showed that convallatoxin is transported by organic anion transporting polypeptide 1B3 (OATP1B3), however, it is not transported by OATP1B1 and Na^+-dependent taurocholate co-transporting polypeptide (NTCP) (Gozalpour *et al.*, 2014b). At the same time, elacridar was found to be a potent inhibitor of OATP1B1-mediated β-estradiol 17-β-D-glucuronide ($E_2$17βG) but did not inhibit OATP1B3 at the concentration of 20 μM (Karlgren *et al.*, 2012a; Karlgren *et al.*, 2012b). This suggests that if dual inhibition on the level of liver influx and efflux is taking place, other influx transporters than OATP1B3 may be involved.

To study the convallatoxin binding sites in human P-gp, nine amino acids were replaced by an alanine. The P-gp residues were selected based on recent refined crystal structure of mouse P-gp and biochemical studies and homology modeling that introduced drug-binding sites of P-gp. Ile306, located in transmembrane helix 5 (TM5) of P-gp, plays an important role in binding of drugs such as verapamil and cyclic peptide inhibitor QZ59-SSS and coupling of drug binding to ATPase activity (Loo & Clarke, 2005; Ravna *et al.*, 2009; Loo & Clarke, 2013; Li *et al.*, 2014). Phe336 and Ile340 are located in hydrophilic face of TM6, the critical TM for P-gp-drug interaction. Both residues are binding sites for QZ59-SSS and QZ59-RRR (Loo *et al.*, 2009; Ravna *et al.*, 2009; Li *et al.*, 2014) and Ile340 also binds to rhodamine (Loo & Clarke, 2002a). In addition, we showed that Phe336 and Ile340 are P-gp interaction sites with DLCs such as cymarin, digoxin, peruvoside, and proscillaridin A (Gozalpour *et al.*, 2013). Like Phe336, Phe343 is one of nine conserved aromatic residues between mouse and human but not C. *elegans* and is a ligand binding residue for rhodamine, QZ59-SSS and QZ59-RRR (Loo *et al.*, 2007; Ravna *et al.*, 2009; Li *et al.*, 2014). Phe728 of TM7 is a drug binding site for verapamil, colchicine, QZ59-SSS and QZ59-RRR (Loo *et al.*, 2006b; Li *et al.*, 2014). Phe942 and The945, located in TM11, have been found as verapamil binding sites (Loo & Clarke, 2002b; Li *et al.*, 2014). Furthermore, Leu975 and Val 982 located in TM12 have been

characterized as drug binding sites for verapamil, colchicine, vinblastine, and QZ59-SSS, additionally; Val982 binds to QZ59-RRR (Loo & Clarke, 2002a; b; Loo *et al.*, 2009; Ravna *et al.*, 2009; Li *et al.*, 2014).

Transport activity of P-gp mutants was investigated using NMQ as a substrate (Hooiveld *et al.*, 2002). The P-gp mutants, I306A, F343A, and F728A exhibited a NMQ transport activity that was less than 30% of the wild type activity. The transport activity of F336A, F942A, T945A, L975A and V982A, were conserved (45-100% of wild type) (Figure 5B) similar to our previous results (Gozalpour *et al.*, 2013). Convallatoxin transport activity of most mutants (I306A, F336A, I340A, F728A, F942A, T945, and L975) was similar to that of NMQ. We conclude that Ile306, Phe336, Phe728, Phe942, Thr945, and Leu975, whose mutation to alanine reduced P-gp transport activity to 50% of wild type, are important residues for interaction with NMQ and convallatoxin.

The type of sugar moiety at position C3 is the single difference between the structures of convallatoxin (L-rhamnose) and cymarin (D-cymarose), whereas unlike cymarin, convallatoxin is transported by P-gp. Interestingly, a single mutation of Ile340 decreased the cymarin inhibition (Gozalpour *et al.*, 2013), but had little effect on the transport of convallatoxin. Consequently, the sugar moiety of DLCs seems to play an important role in the interaction with Ile340 and subtle changes to this moiety can determine if the DLCs are transported by P-gp.

The transport activities of NMQ and convallatoxin were significantly different for the F343A and V982A mutant (Figure 5D and E). NMQ transport activity of F343A was completely diminished, whereas convallatoxin transport activity was conserved. Loo et al. observed that mutation of Phe343 to arginine reduced the affinity of P-gp for rhodamine B and cyclosporine A, but not for vinblastine (Loo *et al.*, 2007). Phe343 seems to play an important role in the transport of NMQ, but is less important for convallatoxin transport. The V982A mutant lost 50% of its convallatoxin transport activity, whereas NMQ transport activity was not changed. This residue had been reported as the binding site for verapamil and rhodamine B (Loo *et al.*, 2006b; 2009). We previously showed that V982A did not influence the affinity of DLCs such as cymarin, digitoxin, digoxin, peruvoside, proscillaridin A and strophanthidol (Gozalpour *et al.*, 2013). As convallatoxin and cymarin only differ in their sugar moiety at position C3, this seems to play a key role in the interaction of convallatoxin with Val982.

It is the first time that the extract of *Convallaria majalis*, convallatoxin, is shown to be transported by P-gp and its transport can be inhibited by elacridar both *in vitro* and *in vivo*. Our results indicate that Val982 is crucial for convallatoxin transport by P-gp, possibly through binding of its sugar moiety. Detailed understanding of the P-gp drug binding sites might aid to select DLCs with better pharmacokinetic profiles that are less prone to drug-drug interactions.

Supplementary Table A. DLCs uptake by P-gp membrane vesicles in the presence of AMP and ATP.

Compounds	Uptake in the presence of AMP	Uptake in the presence of ATP	Ratio (ATP: AMP)
NMQ	0.0090 ± 0.0008 [a]	0.042 ± 0.003 ***	5
convallatoxin	0.062 ± 0.009 [b]	0.38 ± 0.06 ###	6
	0.42 ± 0.06 [c]	2.23 ± 0.11 ***	5
cymarin	0.21 ± 0.07	0.20 ± 0.06	0.9
	1.1 ± 0.1	1.4 ± 0.1	1
digitoxigenin	0.19 ± 0.03	0.15 ± 0.05	0.8
	45.6 ± 13.5	23.5 ± 7.0	0.5
digitoxin	0.66 ± 0.16	0.55 ± 0.12	0.8
	11.4 ± 0.8	5.3 ± 0.6	0.5
digoxigenin	0.060 ± 0.009	0.06 ± 0.02	1
	0.40 ± 0.05	0.23 ± 0.06	0.6
digoxin	0.11 ± 0.01	0.16 ± 0.02	1
	19 ± 2	18.5 ± 3.6	1
dihydroouabain	1.50 ± 0.07	1.50 ± 0.01	1
	1.7 ± 0.1	1.60 ± 0.05	0.9
gitoxigenin	0.040 ± 0.006	0.043 ± 0.003	1
	58 ± 4	54 ± 1	0.9
ouabain	0.012 ± 0.001	0.012 ± 0.001	1
	0.057 ± 0.007	0.093 ± 0.003	1.6
ouabagenin	1.063 ± 0.045	1.14 ± 0.06	1
	0.8 ± 0.2	0.7 ± 0.2	0.9
peruvoside	0.12 ± 0.02	0.13 ± 0.01	1
	0.43 ± 0.07	0.35 ± 0.05	0.8
strophanthidin	0.10 ± 0.02	0.09 ± 0.05	0.9
	0.42 ± 0.07	0.50 ± 0.14	1
strophanthidol	0.08 ± 0.01	0.070 ± 0.006	0.9
	0.37 ± 0.03	0.30 ± 0.03	0.8
proscillaridin A	0.33 ± 0.01	0.35 ± 0.01	1
	0.61 ± 0.02	0.56 ± 0.02	0.9

[a] The uptake at 0.1 μM NMQ, [b] The uptake at 100 μM DLC, [c] The uptake at 1000 μM DLC. The uptake of NMQ and DLCs in P-gp membrane vesicles (nmol mg protein^{-1} min^{-1}) in the presence of AMP and ATP are shown. An unpaired two-sided Student's t-test was used to determine statistically significance of DLCs uptake by membrane vesicles in the absence versus presence of ATP (*** $p < 0.0001$, ### $p = 0.003$).

Chapter 5

Heterogeneous Transport of Digitalis-like Compounds by P-glycoprotein in Vesicular and Cellular Assays

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Abstract

Digitalis-like compounds (DLCs), the ancient medication of heart failure and atrial fibrillation, are characterized by their toxicity and adverse effects. The main mode of action of DLCs, such as the structurally and pharmacokinetically diverse digoxin, digitoxin and ouabain is their inhibitory effect on Na,K-ATPase. Moreover, drug-drug interactions (DDIs) at the levels of absorption and excretion play a key role in their toxicity, hence, knowledge about the transporters involved might prevent these unwanted interactions.

In the present study, the transport of fourteen DLCs with human P-glycoprotein (P-gp; *ABCB1*) was studied using a liquid chromatography-mass spectrometry (LC-MS) quantification method. DLC transport of P-gp overexpressing Madin-Darby canine kidney (MDCK) and immortalized human renal cells (ciPTEC) were compared to vesicular DLC transport.

In a previous study, using membrane vesicles overexpressing P-gp, we identified convallatoxin as a substrate; however, for the other DLCs tested we could not measure transport in this assay, probably because of their high lipophilicity (Gozalpour et al. 2014a). In this study, cellular accumulation assays showed that digitoxin, digoxigenin, strophanthidin and proscillaridin A are also P-gp substrates. These lipophilic compounds could not be identified as P-gp substrates in the vesicular assay, whereas the less lipophilic convallatoxin was not detected as P-gp substrate in the cellular accumulation assays. To observe the functionality of an efflux transporter in the cellular accumulation assay, lipophilic compounds should enter by passive diffusion, whereas the vesicular transport assay is more appropriate for hydrophilic substrates that do not pass the vesicular membrane. In conclusion, we identified digitoxin, digoxigenin, strophanthidin and proscillaridin A as P-gp substrates using cellular accumulation assays and recognized lipophilicity as an important factor in selecting a suitable transport assay.

Introduction

Digitalis-like compounds (DLCs), naturally-originated substances, are ancient medications that are used to treat heart failure, cardiac arrhythmias and atrial fibrillation due to their positive inotropic effect (Rahimtoola, 1996; Gheorghiade *et al.*, 2006; Prassas *et al.*, 2008). Inhibition of Na,K-ATPase by DLCs leads to increased intracellular Na^+ and subsequently Ca^{2+} in cardiac myocytes, which results in stimulation of cardiac contraction (Schatzmann & Rass, 1965). DLCs share a steroid ring as the core structure responsible for their pharmacodynamic properties, with sugar and lactone moieties at positions C3 and C17, respectively (Schonfeld *et al.*, 1985; Prassas *et al.*, 2008). These compounds are categorized as drugs with a narrow therapeutic index that may cause toxicity symptoms, such as dizziness, fatigue, nausea, loss of appetite, vision disturbance and vomiting, when their plasma levels increase (Dick *et al.*, 1991; Belz *et al.*, 2001).

DDIs at absorption and excretion level are the most important factors causing elevated plasma concentrations of DLCs and subsequent toxicities (Rahimtoola, 1996; Gheorghiade *et al.*, 2006). The concentration of digoxin, the most frequently-prescribed DLC, is increased when concomitantly administered with amiodarone, propafenone, verapamil, quinidine, or omeprazole (Pedersen, 1981; Belz *et al.*, 1982; Nademanee *et al.*, 1984; Marcus, 1985; Woodland *et al.*, 1997; Laer *et al.*, 1998; Fromm *et al.*, 1999; Pauli-Magnus *et al.*, 2001; Li *et al.*, 2013). One of the most important reasons for this is inhibition of P-glycoprotein by the above mentioned drugs (De Lannoy *et al.*, 1992; Tanigawara *et al.*, 1992; Schinkel *et al.*, 1995).

P-glycoprotein (P-gp, *ABCB1*), an ATP-dependent efflux transporter, is expressed in the plasma membrane of epithelial cells in tissues playing a significant role in drug disposition. Apical expression of P-gp in kidney and liver enhances drug elimination via urine and bile, respectively. In addition, the presence of P-gp in intestine and brain limits drugs absorption and central nervous system entrance, respectively (Cordon-Cardo *et al.*, 1989; Gottesman & Pastan, 1993).

Transport of digoxin, digitoxin, and ouabain has been extensively studied (De Lannoy *et al.*, 1992; Cavet *et al.*, 1996; Noe *et al.*, 1997; Reichel *et al.*, 1999; Hagenbuch *et al.*, 2000; Kullak-Ublick *et al.*, 2001; Pauli-Magnus *et al.*, 2001; van Montfoort *et al.*, 2002; Funakoshi *et al.*, 2005). However, information about disposition of other DLCs with similar pharmacodynamic properties is lacking. Determination and characterization of the transporters involved in DLC disposition could provide more information about their pharmacokinetic profiles and possible DDIs. *In vitro* transport studies are frequently performed in cell lines over-expressing P-gp. The application of these cellular transport models is limited for compounds that cannot enter the cell via diffusion. Alternatively, isolated membrane vesicles in an inside-out orientation could be used,

allowing the substrates to be internalized by P-gp activity. To mimic the *in vivo* situation more closely, human cell lines expressing influx and efflux transporter simultaneously could be used to study P-gp-mediated efflux (Wilmer *et al.*, 2010). The broad range of transporters present in the latter model, however, limits defining characteristics for individual transporters involved in drug handling. The limitations of each model may lead to different observations when studying P-gp substrate specificities and should be recognized (Taub *et al.*, 2005; Rautio *et al.*, 2006; von Richter *et al.*, 2009).

In this study, transport of fourteen DLCs was studied in a P-gp over-expressing MDCK cell line and ciPTEC, a human renal cell line expressing several influx and efflux transporters including P-gp. Physicochemical properties were calculated to explain the differences in behavior of the DLCs in the drug transporter assays and to provide a tool that can be used in the selection of the appropriate assay to study substrate transport.

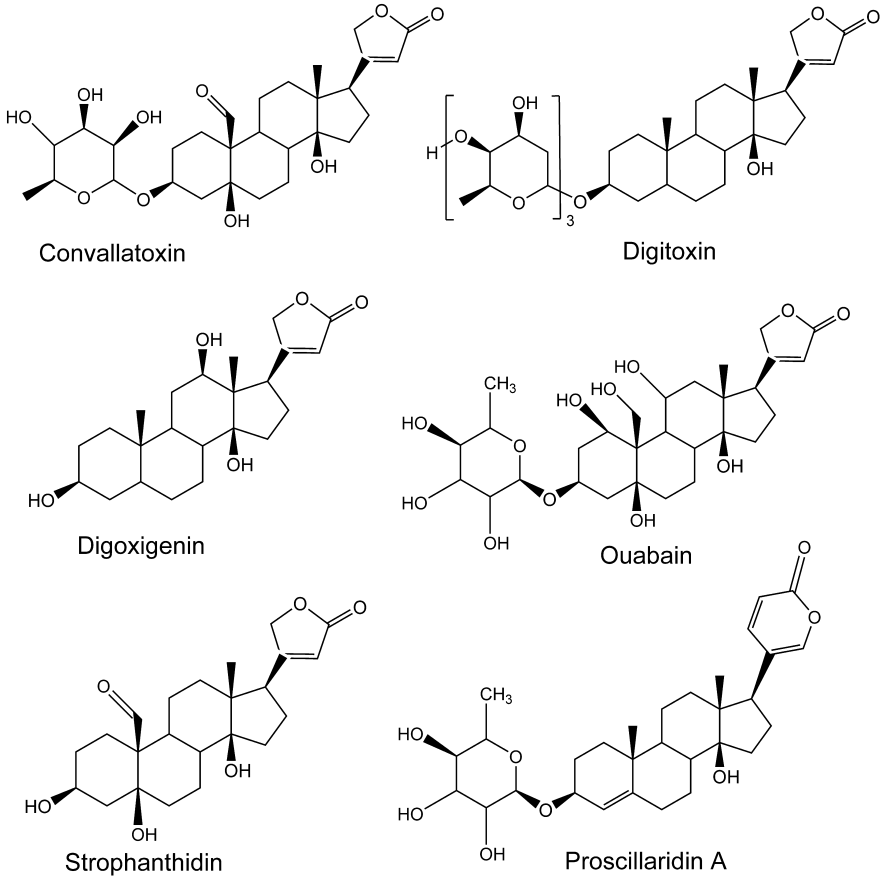

Figure 1. Chemical structures of the digitalis-like compounds convallatoxin, digitoxin, digoxigenin, ouabain, strophanthidin, and proscillaridin A.

Materials and Methods

Materials. [^{3}H]-NMQ (specific activity, 80 Ci/mmol) and unlabeled NMQ were purchased from Solvo Biotechnology (Szeged, Hungary). Convallatoxin (>70% purity), cymarin (>96% purity), digitoxigenin (>98% purity), digitoxin (>96% purity), digoxigenin (>98% purity), digoxin (>95% purity), dihydroouabin (>95% purity), gitoxigenin (>95% purity), ouabagenin (>95% purity), ouabain (>95% purity), peruvoside (90% purity), strophanthidin (>90% purity), strophanthidol (>96% purity), proscillaridin A (>80% purity), elacridar (GF120918), Ko143 were purchased from Sigma (Zwijndrecht, The Netherlands). DMEM high glucose GlutaMAX™ culture medium, DMEM-HAM's F12 medium, Hanks balanced salt solution (HBSS), L-glutamine, sodium pyruvate, 100× penicillin/streptomycin solution were purchased from Life technologies (Breda, The Netherlands). A kit from Bio-Rad Laboratories was used to measure protein concentration (Veenendaal, The Netherlands). Ammonium formate (>99.0 purity) and formic acid (>98% purity) were purchased from Fluka (Steinheim, Germany). DMSO (dimethyl sulfoxide) was obtained from Merck (Darmstadt, Germany) and water was purified with a Millipore® Milli-Q system (Millipore, Bedford, MA, USA). Methanol HPLC grade was purchased from Lab Scan (Dublin, Ireland) and acetonitrile super gradient grade was obtained from VWR (Leuven, Belgium).

Cell culture. Madin-Darby canine kidney (MDCK) and MDCK stably overexpressing human P-gp (MDCK-P-gp) were grown in DMEM high glucose GlutaMAX™. For MDCK cells, that were provide kindly by Dr. M. Gottesman from the National Cancer Institute (Bethesda, USA) (Pastan *et al.*, 1988), the medium was supplemented with 10% FCS, 50 U/mL penicillin and 50 µg/mL streptomycin at 37 °C under 5% CO_2-humidified air.

The human renal conditionally immortalized proximal tubule epithelial cell line (ciPTEC), was previously developed in our lab (Wilmer *et al.*, 2010) and cultured in DMEM-HAM's F12 with supplements as described previously at 33 °C for proliferation of cells. ciPTEC was transferred to 37 °C for 7 days to allow maturation of the renal epithelial phenotype. Both expression and activity of P-gp in matured ciPTEC was demonstrated, using the calcein-accumulation assay.

Cellular accumulation assay. The accumulation of DLCs in MDCK-P-gp, MDCK as the negative control and ciPTEC was measured and compared to determine whether DLCs are transported by P-gp via the cellular accumulation assay. After cell growth in 24-well plates, Tris-HEPES buffer (10 mM HEPES, 132 mM NaCl, 4.2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5.5 mM D-(+)-glucose, adjusted to pH 7.4 using 1 M Tris solution) was used to wash the cells once. Subsequently, the cells were incubated with Tris-HEPES buffer containing 10 µM DLCs at 37 °C in the absence or presence of 2 or

5 μM elacridar (Figures 3 and 4) or 5 μM Ko143 (Figure 3 and 4) for 1 h. After washing cells once with Tris-HEPES containing 0.5% BSA, the cells were washed twice with Tris-HEPES and lysed using 0.5 ml acetonitrile containing 0.1% formic acid for 30 min at room temperature and the protein was precipitated for 30 min at -20 °C.

Sample preparation for LC-MS. Protein-precipitated samples of cellular accumulation assays were centrifuged for 5 min at 16000×g and supernatants were transferred to the new tubes for evaporation under N_2 gas at 37 °C. The samples were completely evaporated and dissolved in methanol containing 200 nM gitoxigenin as an internal standard to determine the concentration of DLCs using LC-MS quantification method. When gitoxigenin was the test compound, ouabain was used as internal standard.

Protein concentration. In all cell accumulation assays, the amount of cellular protein per well was determined in a parallel plate. Distilled water was used to lyse MDCK cells and ciPTEC was lysed in RIPA buffer (1% Igepal CA630, 0.5% Nadeoxycholate, 0.1% SDS, 0.01% phenylmethane sulphonylfluoride, 3% aprotinin and 1 mM Na-orthovanadate) and the cell lysate was stored at -20 °C upon protein concentration analysis using Bio-Rad protein assay kit. DLCs accumulation in ciPTEC and MDCK cells was expressed as nmol per mg protein.

LC-MS quantification of DLCs. DLCs concentrations in the cell lysate samples were measured using the Accela® UPLC (Thermo Scientific, San Jose, CA, USA) coupled to a TSQ Vantage® (Thermo Scientific, San Jose, CA, USA) triple quadrupole mass spectrometer as it was previously described (Gozalpour *et al.*, 2014b). Briefly, the compounds were separated on a HSS T3 analytical column (1.8 μm; 100 × 2.1 mm, Acquity® UPLC, Waters, Ireland) coupled with a VanGuard® HSS T3 pre-column (1.8 μm; 5 × 2.1 mm, Acquity® UPLC, Waters, Ireland). Positive ion mode was used with single ion monitoring (SIM) for the quantitative analysis of fourteen different digitalis-like compounds. The most abundant adducts (sodium $[M+Na]^+$ or potassium $[M+K]^+$ were used for quantification.

Analysis. All the data are expressed as mean ± S.E.M for two or three independent experiments in the cellular accumulation assays. The unpaired Student's t-test was performed to compare groups in the cellular accumulation assays using Graphpad Prism software (version 5.02; Graphpad Software Inc., San Diego, CA).

The physicochemical properties of DLCs, octanol: water partition coefficient (logP) and topological polar surface area (tPSA), were calculated using ChemBioOffice software package (CambridgeSoft, Cambridge, MA, USA) and DLCs molecular weights (MW) were obtained from the Pubchem database (National Centre for Biotechnology Information, Bethesda, MD, USA).

Results

Identification of P-gp substrates in MDCK cells accumulation assay. In a previous study the uptake of fourteen DLCs by P-gp-membrane vesicles was measured and convallatoxin was identified as P-gp substrate (Gozalpour *et al.*, 2014a). However, no significant ATP-dependent uptake of other DLCs was observed. Still, this may not be indicative of the DLCs not being a substrate for P-gp. Therefore, the intracellular accumulation of DLCs in MDCK and MDCK-P-gp cells was measured to determine possible P-gp substrates. In MDCK-P-gp, the accumulation of four DLCs, digitoxin (0.20 ± 0.04 nmol mg protein^{-1}), digoxigenin (0.12 ± 0.03 nmol mg protein^{-1}), strophanthidin (0.160 ± 0.005 nmol mg protein^{-1}) and proscillaridin A (0.19 ± 0.01 nmol mg protein^{-1}) was significantly lower than in MDCK cells not expressing the efflux pump (0.50 ± 0.02, 0.32± 0.05, 0.550 ± 0.009, and 0.460 ± 0.008 nmol mg protein^{-1}, respectively), whereas there was no significant reduction in the accumulation of other DLCs in MDCK-P-gp compared to MDCK cells (Figure 2A-K). In addition, no accumulation of dihydroouabain, ouabain and ouabagenin in the cellular assay was observed (data not shown). As NMQ was previously used as P-gp substrate in the vesicle assays (Gozalpour *et al.*, 2013), we also used it in the cellular accumulation assay. Remarkably, we did not observe a significant difference between NMQ accumulation in MDCK-P-gp and MDCK cells (Figure 2L).

Effect of elacridar and Ko143 on DLC accumulation in MDCK-P-gp. As mentioned in the previous section, digitoxin, digoxigenin, strophanthidin and proscillaridin A were identified as P-gp substrates in the MDCK cellular accumulation assay. We investigated further the effect of the P-gp inhibitor, elacridar, on their intracellular accumulation. The accumulation of digitoxin, digoxigenin, strophanthidin and proscillaridin A in MDCK-P-gp cells was significantly increased compared to control by 10, 5.6, 5.2, and 9 folds, respectively, in the presence of 2 μM elacridar (Figure 3A-D and Table 1). Since elacridar may also inhibit endogenously expressed breast cancer resistance protein (BCRP) (Cooray *et al.*, 2002), the effect of a BCRP inhibitor, Ko143, on DLCs accumulation was also investigated. The presence of 5 μM Ko143 increased the accumulation of digitoxin and proscillaridin A by 2.25 and 1.5 folds, respectively, in MDCK-P-gp compared with MDCK cells. No significant effect was observed for digoxigenin and strophanthidin accumulation in MDCK-P-gp (Figure 3A-D and Table 1).

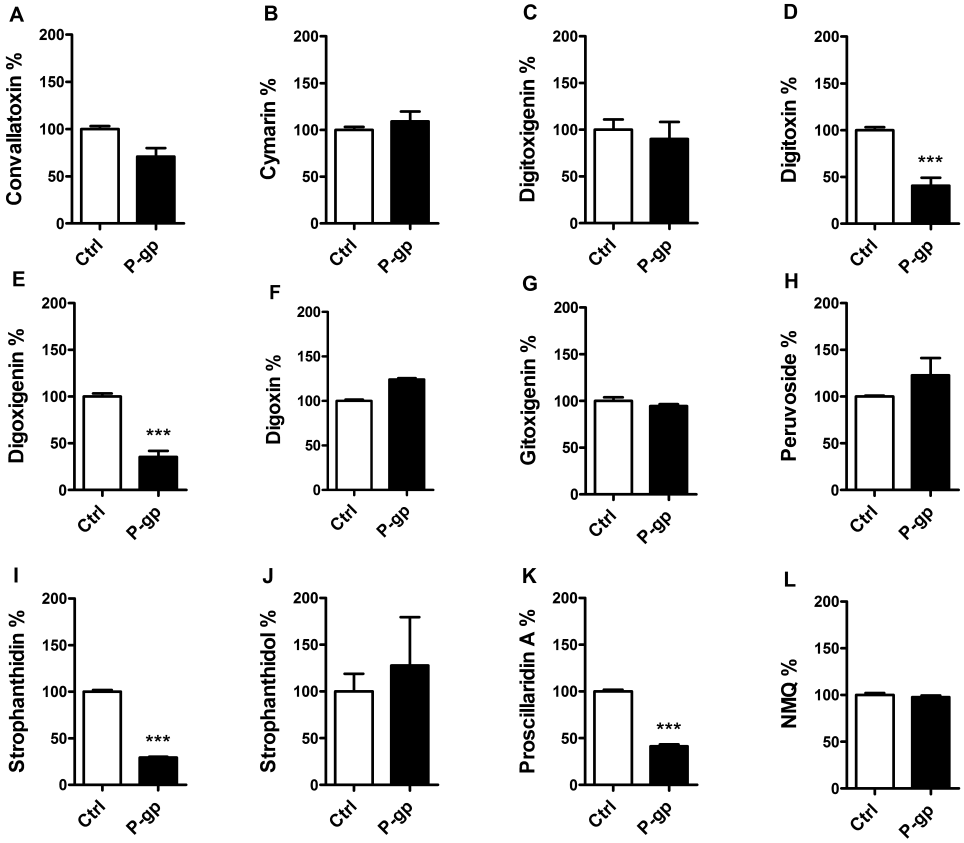

Figure 2. Accumulation of DLCs and NMQ in MDCK and MDCK-P-gp cells. The intracellular accumulation of fourteen DLCs was determined using LC-MS after one hour incubation of MDCK (Ctrl, white bars) and MDCK-P-gp (black bars) with 10 μM convallatoxin (A), cymarin (B), digitoxigenin (C), digitoxin (D), digoxigenin (E), digoxin (F), gitoxigenin (G), peruvoside (H), strophanthidin (I), strophanthidol (J) and proscillaridin A (K). [^{3}H]-NMQ (1 μM) was used to determine the accumulation of NMQ in MDCK and MDCK-P-gp cells after one hour incubation. The mean ± S.E.M (nmol mg protein $^{-1}$) of three experiments are shown and statistically significant differences were determined using an unpaired Student's t-test (***$p < 0.0001$).

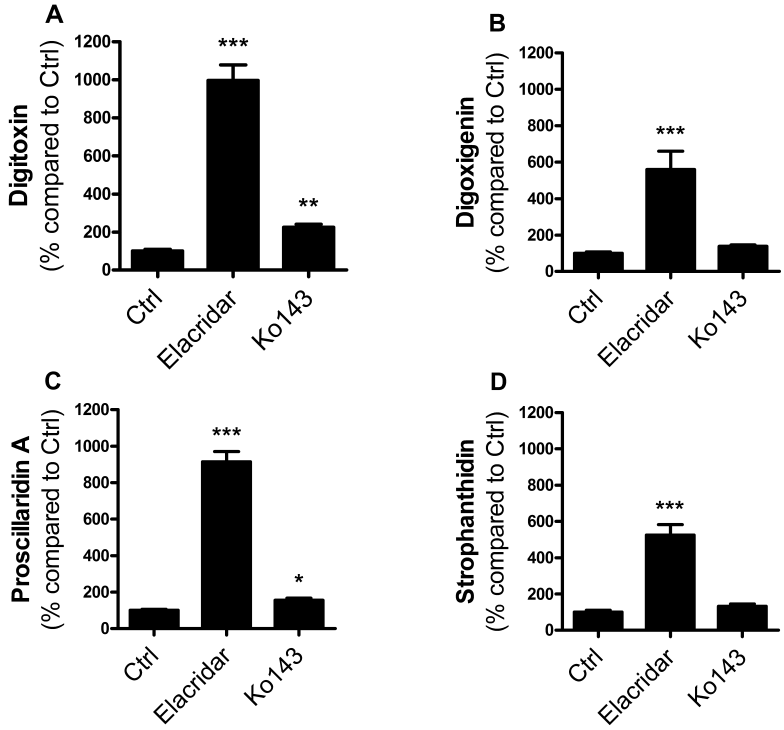

Figure 3. Effect of elacridar and Ko143 on DLC accumulation in MDCK-P-gp cells. Accumulation of digitoxin (A), digoxigenin (B), proscillaridin A (C) and strophanthidin (D) in MDCK-P-gp was determined after one hour incubation of these DLCs (10 μM) with the cells in the absence (Ctrl) and presence of 2 μM elacridar or 5 μM Ko143. The accumulation of DLCs in the absence of inhibitors was set at 100% and an unpaired Student's t-test was used to compare elacridar and Ko143 conditions with those of Ctrl. Mean ± S.E.M of three independent experiments are shown (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.0001$).

DLC accumulation in ciPTEC. The accumulation of selected DLCs, convallatoxin, digoxin, ouabain, strophanthidin and proscillaridin A was analyzed in ciPTEC, in presence and absence of elacridar and Ko143. These DLCs were selected based on the observations in the cellular accumulation and vesicular transport assays (Gozalpour *et al.*, 2014a). Digoxin, strophanthidin and proscillaridin A, were demonstrated to accumulate in the presence of elacridar (2.2, 1.5, and 2.0 fold increase, respectively) (Figure 4 and Table 1). Moreover, Ko143 increased accumulation of digoxin, strophanthidin and proscillaridin A by 1.7, 1.5, and 1.6 folds, respectively (Figure 4 and Table 1). Convallatoxin, which was identified as a P-gp substrate in the vesicular transport assay, did not accumulate in ciPTEC in presence of elacridar or Ko143 (Figure 4A and Table 1). In addition, ouabain, which was not a P-gp substrate in both cellular and vesicular assays, did not accumulate in ciPTEC either (data not shown).

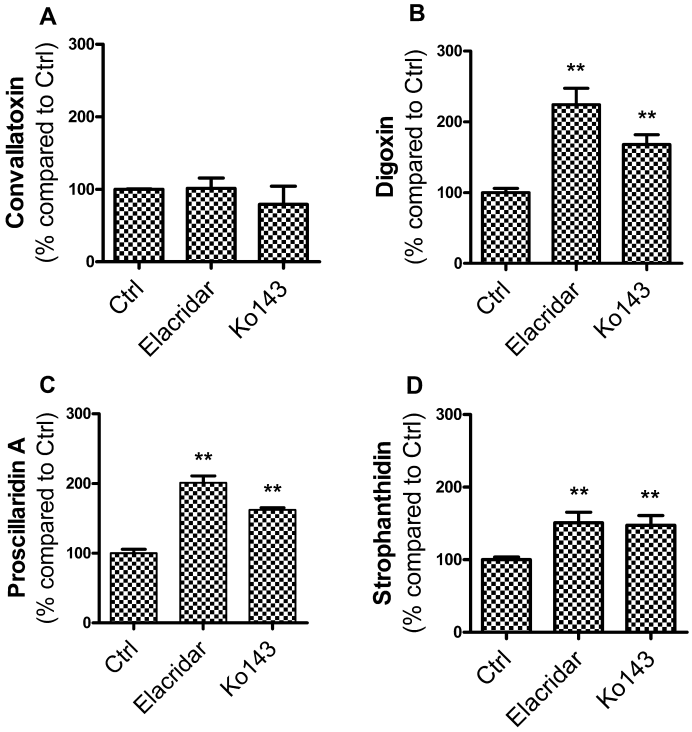

Figure 4. Effect of elacridar and Ko143 on DLC accumulation in ciPTEC. Accumulation of convallatoxin (A), digoxin (B), proscillaridin A (C), and strophanthidin (D) in ciPTEC was determined after one hour incubation of these DLCs (10 μM) with the cells in the absence (Ctrl) and presence of 5 μM elacridar or Ko143. The accumulation of DLCs in the absence of inhibitors was set at 100% and an unpaired Student's t-test was used to compare elacridar and Ko143 conditions with those of Ctrl. Mean ± S.E.M of two independent experiments are shown (** $p <$ 0.01).

Table 1. Accumulation of DLCs in MDCK-P-gp and ciPTEC in the absence and presence of inhibitors.

Compounds	**Cellular Accumulation (nmol mg protein^{-1})** [a]				
	MDCK-P-gp			**ciPTEC**	
	Ctrl	**Elacridar**	**Ko143**	**Ctrl**	**Elacridar**
convallatoxin				46.1 ± 0.3	29 ± 8
digitoxin	0.19 ± 0.02	1.87 ± 0.15	0.423 ± 0.003		
digoxigenin	0.13 ± 0.01	0.71 ± 0.13	0.18 ± 0.01		
digoxin				10 ± 4	19 ± 8
strophanthidin	0.17 ± 0.02	0.9 ± 0.1	0.23 ± 0.02	27 ± 8	45 ± 16
proscillaridin A	0.23 ± 0.01	2.1 ± 0.13	0.35 ± 0.03	7.1 ± 0.4	14.3 ± 0.7

[a] The intracellular accumulation (nmol mg protein^{-1}) of four DLCs in MDCK-P-gp and ciPTEC in the absence and presence of elacridar (2 μM in MDCK-P-gp and 5 μM in ciPTEC) and 5 μM Ko143 was determined using LC-MS quantification after one hour incubation of cell lines with 10 μM DLCs. The mean ± S.E.M of two experiments in triplicate are shown.

Comparison of physicochemical properties of P-gp substrates. To compare the results of P-gp vesicular transport assay and the cellular accumulation assays, physicochemical properties of DLCs such as molecular weight (MW), lipophilicity as calculated logP (clogP), and topological polar surface area (tPSA) were compared (Table 2). Of the P-gp substrates, convallatoxin with a clogP of -0.7 was the least lipophilic, whereas, digitoxin and proscillaridin A, were the most lipophilic P-gp substrates (with clogP values of 2.9 and 2.6, respectively) (Table 2).

To confirm whether DLC lipophilicity influences the results of vesicular transport and cellular accumulation assays, the observed accumulation of fourteen DLCs in MDCK was plotted against calculated logP, molecular weight and calculated polar surface area. No significant correlation was found between cellular accumulation and molecular weight or polar surface area (data was not shown), but a significant correlation was found between the clogP and accumulation of DLCs in MDCK cells ($r^2 = 0.32$, $p = 0.03$) (Figure 5) indicating that lipophilic DLCs accumulate better in MDCK cells than hydrophilic congeners. Moreover, as gitoxigenin is highly accumulated in MDCK cells, if gitoxigenin was excluded from the graph, the r^2 and p values increased to 0.53 and 0.005, respectively.

Table 2. P-gp transport characteristics and calculated physicochemical properties of DLCs.

Compounds	P-gp-mediated transport [a]			MW [b]	clogP [c]	tPSA [d]
	Membrane vesicle	MDCK	ciPTEC			
convallatoxin	+	-	-	550.6	-0.7	163.0
cymarin	-	-	nd	548.7	0.2	131.8
digitoxigenin	-	-	nd	374.5	2.5	66.8
digitoxin	-	+	nd	765.0	2.9	182.8
digoxigenin	-	+	nd	390.5	1.1	87.0
digoxin	-	-	+	781.0	1.4	203.6
dihydroouabain	-	-	nd	586.7	-1.8	206.6
gitoxigenin	-	-	nd	390.5	2.3	87.0
ouabain	-	-	nd	584.7	-1.7	206.6
ouabagenin	-	-	nd	438.5	-1.3	147.7
peruvoside	-	-	nd	548.7	0.3	131.8
strophanthidin	-	+	+	404.5	-0.3	104.1
strophanthidol	-	-	nd	406.5	-0.28	107.2
proscillaridin A	-	+	+	530.7	2.6	125.7

[a] P-gp substrates and non-substrates in each assay are shown by (+) and (-), respectively.
[b] Molecular weight, [c] Calculated octanol: water partition coefficient, [d] Calculated polar surface area and nd, not determined

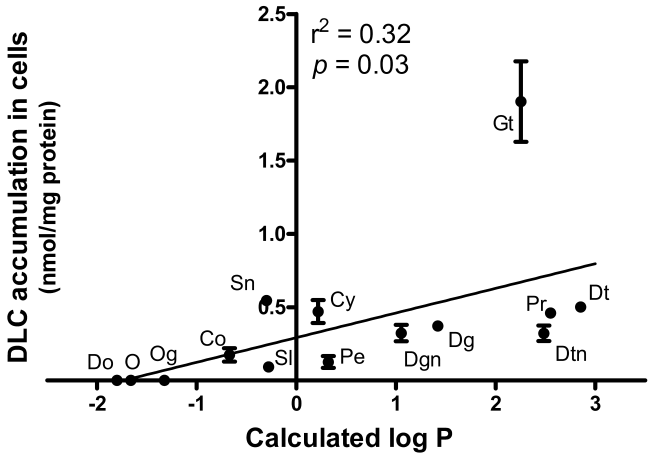

Figure 5. Relationship between cellular accumulation of DLCs and their lipophilicity. The accumulation in MDCK cells was plotted against calculated logP values of DLCs ($p < 0.05$). Do, dihydroouabain, O, ouabain, Og, ouabagenin, Co, convallatoxin, Sn, strophanthidin, Sl, strophanthidol, Cy, cymarin, Pe, peruvoside, Dgn, digoxigenin, Dg, digoxin, Gt, gitoxigenin, Pr, proscillaridin A, Dtn, digitoxigenin and Dt, digitoxin.

Discussion

In the present study, P-gp-mediated transport of fourteen DLCs was determined using cellular accumulation assays. We previously identified convallatoxin as a P-gp substrate in the vesicular transport assay (Gozalpour *et al.*, 2014a) and in this study we identified four more DLCs to be substrates of P-gp in two different cellular accumulation assays. The results of these three assays were compared with the physicochemical properties of the DLCs used. Our data confirm that clogP of the compounds should be taken into account when selecting a drug transporter assay. Digitoxin and digoxigenin were shown to be P-gp substrates, which is in line with other studies showing that digitoxin and digoxigenin are transported by P-gp in LLC-PKI and Caco-2 cell lines (Pauli-Magnus *et al.*, 2001; Hughes & Crowe, 2010). However, this is the first study that identifies proscillaridin A and strophanthidin as P-gp substrates. Based on the expression of P-gp in canalicular membranes of hepatocytes (Thiebaut *et al.*, 1987), our study elucidates a role for P-gp and probably BCRP in biliary excretion of digitoxin, strophanthidin and proscillaridin A that were shown to be excreted via liver.(Andersson *et al.*, 1977a; Andersson *et al.*, 1977b; Strobach *et al.*, 1986; Belz *et al.*, 2001).

In the current study, digitoxin, digoxigenin, strophanthidin and proscillaridin A were demonstrated to be P-gp substrates because their accumulation in MDCK-P-gp was significantly less than control MDCK cells. Whereas digitoxin and proscillaridin A were also identified as inhibitors, digoxigenin and strophanthidin did not or hardly inhibit P-gp transport, most likely reflecting their low P-gp affinity (Gozalpour *et al.*, 2013). In addition, the accumulation of these four DLCs was increased by the P-gp inhibitor elacridar. Accumulation of digitoxin and proscillaridin A in MDCK-P-gp was also reduced by Ko143, which suggests that these two DLCs are also transported by endogenous BCRP. It has, however, been reported that MDCK cells are lacking BCRP expression (Quan *et al.*, 2012; Gartzke & Fricker, 2014). Moreover, digoxin, strophanthidin and proscillaridin A accumulation was also increased in ciPTEC in presence of Ko143, also suggesting the involvement of BCRP in efflux of these DLCs, which is underscored by endogenous BCRP activity present in ciPTEC (Jansen *et al.*, 2014).

In contrast to the results of vesicular transport assay, convallatoxin and NMQ were not identified as P-gp substrates in the cellular accumulation assays. On the other hand, there was no evidence of P-gp-mediated transport of digitoxin, digoxigenin, strophanthidin and proscillaridin A by P-gp in the vesicular transport assay (Gozalpour *et al.*, 2014a). This shows that assay properties affect the identification of P-gp substrates. Interestingly, convallatoxin, the only P-gp substrate identified in the vesicular transport assay, was ranked least lipophilic after dihydroouabain, ouabain, and ouabagenin of all DLCs tested. Previously, we identified OATP1B3 as the transporter of convallatoxin,

dihydroouabain, ouabain, and ouabagenin (Gozalpour *et al.*, 2014b). The absence of OATP1B3 in MDCK cell lines (Goh *et al.*, 2002; Quan *et al.*, 2012) and ciPTEC explain explains the lack of convallatoxin uptake and subsequent efflux by P-gp in the cellular assays. Unlike the cellular accumulation assay, the inside-out vesicular transport assay is suitable to measure transport of low permeable (hydrophilic) compounds (Szeremy *et al.*, 2011). Digitoxin and proscillaridin A, the most lipophilic DLCs, were transported in the cellular accumulation assay but not in the vesicular transport assay. Passive diffusion of these lipophilic DLCs into vesicles may lead to a high background that conceals P-gp-mediated uptake. The cellular accumulation assay is suitable for medium and high permeable compounds, because P-gp substrates require crossing the cell membrane by diffusion to interact with the efflux transporter, provided no influx transporters are present (Figure 6) (Szeremy *et al.*, 2011). P-gp substrates could be categorized based on their clogP as follows: convallatoxin (clogP: - 0.7) < strophanthidin (clogP: - 0.3) < digoxigenin (clogP: 1.1) < digoxin (clogP: 1.4) < proscillaridin A (clogP: 2.6) < digitoxin (clogP: 2.9). As P-gp-mediated strophanthidin transport could be detected in cellular assays and convallatoxin transport not, we suggest a threshold clogP value of - 0.3 to measure transport of DLCs in a cellular accumulation assay. The significant relationship between the lipophilicity of DLCs and their cellular accumulation in MDCK cells is in line with studies showing high intestinal absorption of lipophilic DLCs (Haass *et al.*, 1972; Mooradian, 1988).

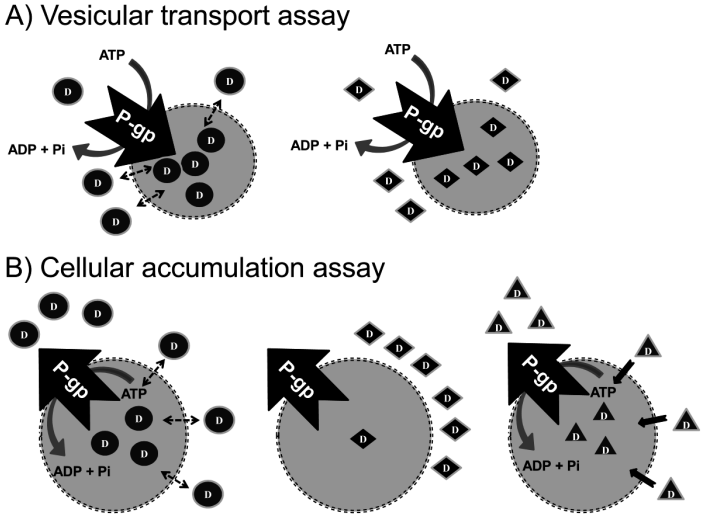

Figure 6. Behavior of DLCs in vesicular transport and cellular accumulation assays. P-gp mediated transport of lipophilic DLCs (black circle) and hydrophilic DLCs (black diamond) are shown in vesicular transport (A) and cellular accumulation (B) assays. DLCs which need an influx transporter (black one-way arrows) are shown by triangles in the cellular assay, black dotted arrows represent passive diffusion of DLCs.

Surprisingly, digoxin was only characterized as a P-gp substrate in ciPTEC and not in the MDCK-P-gp cells. Two factors could explain this observation. First, we sequenced the P-gp cDNA present in the MDCK-P-gp cell line and observed that the Gly185Val mutation present in the originally cloned P-gp was also present in this cell line (data not shown). It is known that this mutation affects the substrate affinity (Safa *et al.*, 1990; Watanabe *et al.*, 2000) and therefore might affect the digoxin transport. Secondly, only in ciPTEC, accumulation of digoxin could be demonstrated in the presence of elacridar and Ko143. It is known that influx of digoxin is enhanced by expression of influx transporters, such as the Solute Carrier Organic Anion Transporter (SLCO4C1, OATP4C1) (Mikkaichi *et al.*, 2004). Previously, we demonstrated that SLCO4C1 mRNA is present in ciPTEC (Jansen *et al.*, 2014) whereas, it has not been detected in MDCK cells (Goh *et al.*, 2002; Szeremy *et al.*, 2011). In addition to OATP4C1 at the basolateral membrane of the kidney, a Na-dependent uptake transporter at basolateral membrane of liver and kidney has been reported as digoxin uptake transporter (Taub *et al.*, 2011). The presence of digoxin uptake transporters and their variable expression in different cell lines might explain the high variability in the measurement of net digoxin efflux in different cell lines (Lee *et al.*, 2014).

In conclusion, we assessed P-gp-mediated transport of fourteen DLCs in cellular transport assays and compared the results to that of a previous vesicular transport assay. Our results show that digitoxin, digoxigenin, strophanthidin, and proscillaridin A, like convallatoxin in the vesicular transport assay, are P-gp substrates. The importance of DLCs lipophilicity in the selection and outcome of the transport assay was confirmed and we conclude that DLCs with a clogP of - 0.3 and higher should be used in cellular assays.

Chapter 6

Interaction of Digitalis-Like Compounds with Liver Uptake Transporters NTCP, OATP1B1, and OATP1B3

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Abstract

Digitalis-like compounds (DLCs) such as digoxin, digitoxin, and ouabain, also known as cardiac glycosides, are among the oldest pharmacological treatments for heart failure. The compounds have a narrow therapeutic window, while at the same time; DLC pharmacokinetics is prone to drug-drug interactions at the transport level. Hepatic transporters organic anion transporting polypeptide (OATP)1B1, OATP1B3, and Na^+-dependent taurocholate co-transporting polypeptide (NTCP) influence the disposition of a variety of drugs by mediating their uptake from blood into hepatocytes. The interaction of digoxin, digitoxin, and ouabain with hepatic uptake transporters has been studied before. However, here, we systematically investigated a much wider range of structurally related DLCs for their capability to inhibit or to be transported by these transporters in order to better understand the relation between the activity and chemical structure of this compound type.

We studied the uptake and inhibitory potency of a series of 14 structurally related DLCs in Chinese hamster ovary cells expressing NTCP (CHO-NTCP) and human embryonic kidney cells expressing OATP1B1 and OATP1B3 (HEK-OATP1B1 and HEK-OATP1B3). The inhibitory effect of the DLCs was measured against taurocholic acid (TCA) uptake in CHO-NTCP cells and against uptake of β-estradiol 17-β-D-glucuronide ($E_2$17βG) in HEK-OATP1B1 and HEK-OATP1B3 cells.

Proscillaridin A was the most effective inhibitor of NTCP-mediated TCA transport (IC_{50} = 22 μM), whereas digitoxin and digitoxigenin were the most potent inhibitors of OATP1B1 and OAPTP1B3, with IC_{50} values of 14.2 and 36 μM, respectively. Additionally, we found that the sugar moiety and hydroxyl groups of the DLCs play different roles in their interaction with NTCP, OATP1B1, and OATP1B3. The sugar moiety decreases the inhibition of NTCP and OATP1B3 transport activity, whereas it enhances the inhibitory potency against OATP1B1. Moreover, the hydroxyl group at position 12 reinforces the inhibition of NTCP but decreases the inhibition of OATP1B1 and OATP1B3. To investigate whether DLCs can be translocated, we quantified their uptake in transporter-expressing cells by LC-MS. We demonstrated that convallatoxin, ouabain, dihydroouabain, and ouabagenin are substrates of OATP1B3. No transport was observed for the other compounds in any of the studied transporters.

In summary, this work provides a step toward an improved understanding of the interaction of DLCs with three major hepatic uptake transporters. Ultimately, this can be of use in the development of DLCs that are less prone to transporter-mediated drug-drug interactions.

Introduction

Digoxin belongs to a large family of naturally derived compounds called digitalis-like compounds (DLCs). The DLCs are the oldest cardiac medications that have been prescribed because of their inotropic effect (Poole-Wilson & Robinson, 1989). By inhibiting Na,K-ATPase, DLCs induce an increase in intracellular Na^+ concentration followed by an increased intracellular Ca^{2+} concentration in cardiomyocytes, leading to a stronger contraction of the heart muscle (Ahmed *et al.*, 2008; Prassas & Diamandis, 2008). Currently, digoxin is applied for the treatment of heart failure and arrhythmia (DIG, 1997; Gheorghiade & Pitt, 1997); however, it has a narrow therapeutic window. Supra-therapeutic concentrations of digoxin in plasma rapidly lead to a range of adverse effects ranging from anorexia, fatigue, nausea, vomiting, and visual disturbances to ventricular fibrillation and ultimately death (Vivo *et al.*, 2008).

Digoxin is partly metabolized in the liver, and digitoxin is mainly cleared via the hepatic route (Okita *et al.*, 1955a; b; Doherty & Kane, 1975). Drug−drug interactions (DDIs) that result in lower clearance of digoxin from the body are among the most important factors causing toxicity. The majority of bioavailable digoxin (60−80%) is excreted via the kidney as unchanged drug, whereas the rest is metabolized and excreted via the liver (Hanratty *et al.*, 2000). Inhibition of P-glycoprotein (P-gp), the efflux transporter of digoxin and digitoxin (de Lannoy & Silverman, 1992; Pauli-Magnus *et al.*, 2001a), by coadministered medications such as quinidine, cyclosporine, and amiodarone leads to increased blood levels, causing toxicity (Belz *et al.*, 1983; Warner *et al.*, 1985; Robinson *et al.*, 1989; de Lannoy & Silverman, 1992; Robieux *et al.*, 1992; Hanratty *et al.*, 2000; Vivo *et al.*, 2008).

Next to the cellular efflux of DLCs mediated by P-gp, cellular influx through SLC transporters may play a role in the disposition of these compounds in the liver and kidney. Among the hepatic uptake transporters, two members of the organic anion transporting polypeptide (OATP) family, OATP1B1 (SLCO1B1) and OATP1B3 (SLCO1B3), are predominantly expressed in human liver (Hsiang *et al.*, 1999; Konig *et al.*, 2000a; b). Moreover, Na^+-dependent taurocholate co-transporting polypeptide (SLC10A1, NTCP) is an uptake transporter of bile salts and a number of other substrates with a steroid-like structure (Hagenbuch & Meier, 1994; Meier *et al.*, 1997; Alrefai & Gill, 2007). Next to digoxin and digitoxin, many structurally related DLCs exist, yet the excretion route of these compounds is unknown. The majority of naturally occurring DLCs have not been investigated with regard to their interaction with hepatic uptake transporters. In fact, only the hepatic uptake of the cardiac glycosides digoxin and ouabain has been well-characterized in mouse, rat, and human. In rats, Oatp2 and Oatp4 were found to be hepatic uptake transporters (Noe *et al.*, 1997; Cattori *et al.*, 2000; Hagenbuch *et*

al., 2001; Sugiyama *et al.*, 2002; Funakoshi *et al.*, 2005; Ose *et al.*, 2010). In addition, murine and rat Oatp1 and Oatp2 were reported as ouabain uptake transporters (Meier *et al.*, 1997; Reichel *et al.*, 1999; Hagenbuch *et al.*, 2000; van Montfoort *et al.*, 2002). In human hepatocytes, digoxin uptake was shown to be transporter-dependent (Olinga *et al.*, 1998; Kimoto *et al.*, 2011; De Bruyn *et al.*, 2013); moreover, digoxin and ouabain were reported as OATP1B3 substrates using *Xenopus laevis* oocytes (Kullak-Ublick *et al.*, 2001) and polymorphisms in OATP1B3 have been suggested to be associated with increased concentrations of digoxin in plasma (Tsujimoto *et al.*, 2008a). However, the same result was not obtained by Taub *et al.* using OATP2-transfected cell lines (Taub *et al.*, 2011). Finally, it has been described that NTCP plays no role in ouabain transport, yet NTCP-mediated transport of Bamet could be inhibited by ouabain, indicating that the compound is capable of interacting with this transporter (Kouzuki *et al.*, 1998; Briz *et al.*, 2002).

Replacement of currently applied DLCs with alternative compounds that are less prone to drug-drug interactions occurring at the influx and efflux transporter level may be facilitated by charting the interactions of this compound class with various influx and efflux transporters (Shitara *et al.*, 2005; Giacomini *et al.*, 2010; Gozalpour *et al.*, 2013). In previous work, we addressed the interaction of a range of DLCs with P-gp.

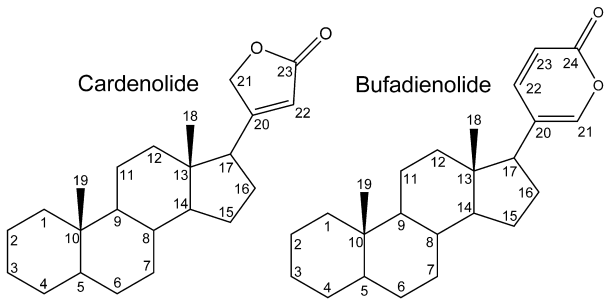
In the present study, we investigated the interaction of 14 DLCs (Figure 1) with human NTCP, OATP1B1, and OATP1B3 using cells overexpressing these transporters. First, we studied the inhibitory potency of DLCs on transporter activity and characterized structural features that are typical for inhibition of each transporter. We also determined if the DLCs were actually transported, and we identified convallatoxin, ouabain, dihydroouabain, and ouabagenin as new substrates for OATP1B3.

Materials and Methods

Materials. High-glucose Dulbecco's modified Eagle's medium (DMEM) (NEAA, no glutamine), GlutaMAX high glucose DMEM, Hanks balanced salt solution (HBSS), L-glutamine, and sodium pyruvate were purchased from Life Technologies (Breda, The Netherlands). [6,7-^{3}H(N)]Estradiol 17-β-D-glucuronide (34.3 Ci/mmol) and [^{3}H(G)]taurocholic acid (5 Ci/mmol) were purchased from PerkinElmer Life and Analytical Sciences (Groningen, The Netherlands). Fetal calf serum was purchased from Greiner (Alphen a/d Rijn, The Netherlands), and BD BioCoat poly-D-lysine 24-well plates were purchased from VWR (Leuven, Belgium). The protein concentrations were determined using a protein assay kit from Bio-Rad Laboratories (Veenendaal, The Netherlands). Convallatoxin (>70% purity), proscillaridin A (>80% purity), peruvoside

and strophanthidin (>90% purity), cymarin, digitoxin, digoxin, dihydroouabain, gitoxigenin, ouabagenin, ouabain and strophanthidol (>96% purity), digitoxigenin and digoxigenin (>98% purity), and sodium butyrate were purchased from Sigma (Zwijndrecht, The Netherlands). Ammonium formate and formic acid were purchased from Fluka (Steinheim, Germany). Dimethyl sulfoxide (DMSO) was obtained from Merck (Darmstadt, Germany), and water was purified with a Millipore Milli-Q system (Millipore, Bedford, MA, USA). HPLC grade methanol was purchased from Lab Scan (Dublin, Ireland), and super gradient grade acetonitrile was obtained from VWR (Leuven, Belgium).

A



B

cardenolides	1	3	5	11	12	14	16	19	Other
convallatoxin		L-rhamnose	OH			OH		O	
cymarin		D-cymarose	OH			OH		O	
digitoxigenin		OH				OH			
digitoxin		tri-D-digitoxose				OH			
digoxigenin		OH			OH	OH			
digoxin		tri-D-digitoxose			OH	OH			
dihydroouabain	OH	L-rhamnose	OH	OH		OH		OH	20-22 saturated
gitoxigenin		OH				OH	OH		
ouabagenin	OH	OH	OH	OH		OH		OH	
ouabain	OH	L-rhamnose	OH	OH		OH		OH	
peruvoside		3-O-methyl glucose				OH		O	
strophanthidin		OH	OH			OH		O	
strophanthidol		OH	OH			OH		OH	
bufadienolides									
proscillaridin A		L-rhamnose	4-5 double bond			OH			

Figure 1. Structural features of DLCs. The cardenolides and bufadienolides contain γ-butyrolactone (γ-lactone) and δ-valerolactone (δ-lactone) at the 17 position, respectively (A). DLCs have a steroid ring as their core structure that contains different chemical substitutions (B).

Cell culture. Chinese hamster ovary cells stably expressing NTCP (CHO-NTCP) were obtained from Solvo Biotechnologies (Budapest, Hungary), and human embryonic kidney cells (HEK293) stably expressing OATP1B1 or OATP1B3 (HEKOATP1B1 and HEK-OATP1B3) were kindly provided by TNO (Zeist, The Netherlands). For all experiments, CHO-parent (CHO-P) and HEK-parent (HEK-P) were used as the

controls. CHO cells were cultured in high-glucose DMEM supplemented with 10% FCS, 4 mM L-glutamine, and 1 mM sodium pyruvate. HEK cells were cultured in GlutaMAX high glucose DMEM supplemented with 10% FCS at 37 °C under 5% CO_2 humidified air.

Kinetic characterization of NTCP, OATP1B1, and OATP1B3. CHO and HEK cells were seeded at a density of 600,000 cells per well (Greiner, Alphen a/d Rijn, The Netherlands) and 400,000 cells per poly-D-lysine-coated well (VWR, Leuven, Belgium) in 24-well plates, respectively. To induce NTCP protein expression, 5 mM sodium butyrate was added to CHO cells. Twenty-four hours after CHO and 48 h after HEK cells were seeded, the medium was removed and the cells were washed twice with HBSS containing 10 mM HEPES (HBSS-HEPES), pH 7, at 37 °C. Subsequently, CHO and HEK cells were incubated with 150 µl of HBSS-HEPES containing [^{3}H]-labeled taurocholic acid or [^{3}H]estradiol 17-β-D-glucuronide, respectively, at 37 °C for the incubation times indicated in the figure legends. To stop the incubation, the cells were placed on ice and washed twice with ice-cold HBSS-HEPES containing 0.5% BSA followed by washing with ice-cold HBSS-HEPES. Next, the CHO and HEK cells were lysed using 1 M NaOH and 0.5% Triton X-100 for 1 h, respectively. After homogenizing, lysed cells were mixed with 4 mL of scintillation liquid, and cell-associated radioactivity was measured using a Packard Tri-Carb liquid scintillation counter (Ramsey, MN, USA). In all experiments, transporter-dependent uptake was calculated by subtracting the values measured using parent cells from the corresponding values measured in CHO and HEK cells expressing transporters. All experiments were performed in triplicate.

Inhibition studies. To screen DLCs for inhibitory effects against NTCP, OATP1B1, and OATP1B3, the CHO and HEK cells were incubated with HBSS-HEPES containing trace amounts of [^{3}H]-labeled taurocholic acid (0.03 µM) and [^{3}H]estradiol 17-β-D-glucuronide (0.0043 µM), respectively. Experiments were performed at 37 °C in the presence of the various DLCs at 10 and 100 µM. Stock solutions of DLCs were made in DMSO, and the final concentration of DMSO was 1% in all incubates. The incubation solutions were supplemented with unlabeled estradiol 17-β-D-glucuronide or taurocholic acid to the concentrations indicated in the figure legends. In addition, 1% DMSO and 100 µM TCA (for NTCP) or 100 µM $E_2$17βG (for OATP1B1 and OATP1B3) were used as negative and positive controls, respectively. Furthermore, the IC_{50} of DLCs on NTCP-mediated TCA transport, OATP1B1-mediated $E_2$17βG transport, and OATP1B3-mediated $E_2$17βG transport was determined by coincubation with increasing concentrations of DLCs (Figure 4).

DLC uptake assays. To determine whether the DLCs are transported by NTCP, OATP1B1, and OATP1B3, the uptake of DLCs was determined in CHO-NTCP, HEK-OATP1B1, and HEK-OATP1B3 as well as in CHO-P and HEK-P as negative controls.

The CHO and HEK cells were incubated with HBSS-HEPES containing 10 μM DLCs at 37 °C for 1 h at a 1% final concentration of DMSO in the absence and presence of the inhibitor rifampicin (100 μM). After the previously described washing steps with HBSS-HEPES (without and with 0.5% BSA), the cells were lysed using 0.5 mL of 99.9% acetonitrile and 0.1% formic acid for 30 min. After addition of the acetonitrile/formic acid mixture, proteins were allowed to precipitate for 30 min at -20 °C. After 5 min of centrifugation at 16000g, the supernatants were transferred to new tubes and the acetonitrile/formic acid was evaporated under a gentle stream of N_2 gas at 37 °C. The pellets were dissolved in methanol containing 200 nM gitoxigenin as an internal standard to analyze the samples using LC−MS. Ouabain was used standard when gitoxigenin was the test compound.

Protein Concentration. In all inhibition and accumulation assays, the amount of cellular protein per well was determined in a parallel plate. Cells were lysed using distilled water and stored -20 °C upon analysis. Protein concentrations were determined using the Bio-Rad protein assay kit. The uptake of substrates in CHO and HEK cells was expressed as picomoles per milligram of protein.

LC-MS Quantification of DLCs. The concentration of DLCs in the cell lysate samples was measured using an Accela UPLC (Thermo Scientific, San Jose, CA, USA) coupled to a TSQ Vantage (Thermo Scientific, San Jose, CA, USA) triple quadrupole mass spectrometer. The compounds were separated on a HSS T3 analytical column (1.8 μm; 100 × 2.1 mm, Acquity UPLC, Waters, Ireland) coupled with a VanGuard HSS T3 precolumn (1.8 μm; 5 × 2.1 mm, Acquity UPLC, Waters, Ireland). The mobile phase consisted of solvent A (10 mM ammonium formate, pH 3.0) and solvent B (methanol). The elution gradient was 0 min, 90% A; 0.5 min, 90% A; 12.5 min, 15% A; 13.5 min, 90% A; and 20 min. 90% A for convallatoxin, cymarin, digitoxigenin, digoxigenin, dihydroouabain, gitoxigenin, ouabagenin, ouabain, peruvoside, strophanthidin, and strophanthidol and 0 min, 70% A; 0.5 min, 70% A; 12.5 min, 15% A; 13.5 min, 70% A; and 20 min, 70% A for digitoxin, digoxin, gitoxigenin, and proscillaridin A. Gitoxigenin as the internal standard was measurable under both conditions. The column temperature was set at 60 °C, and the flow rate was 500 μl/min. In addition, the sample injection volume was 20 μl, the analysis run time was 20 min, and the samples were stored at a tray temperature of 8 °C. The effluent from the UPLC was passed directly into the heated electrospray ion source. The capillary temperature and the vaporizer temperature were set at 225 and 382 °C, respectively. With ionization voltage at +3.5 kV, heated electrospray ionization (HESI) was achieved using nitrogen gas as the sheath and auxiliary gases, with pressures of 30 and 35 AU (Arbitrary Units), respectively.

Positive ion mode was used with single ion monitoring (SIM) for the quantitative analysis of 14 different DLCs. The most abundant adducts (sodium $[M+Na]^+$ or potassium $[M+K]^+$) were used for quantification. Adducts with their corresponding mass/charge ratio (m/z) are shown in Table 1.

Table 1. Mass Fragment of most abundant adducts for the detection of DLCs by LC−MS

Compounds	[M+Na]+ (m/z)	[M+K]+ (m/z)
convallatoxin	573.3	
cymarin	571.3	
digitoxigenin	397.2	
digitoxin	787.5	
digoxigenin		492.2
digoxin	803.4	
dihydroouabain	609.3	
gitoxigenin		429.3
ouabain	461.2	
ouabagenin	607.3	
peruvoside	571.2	
strophanthidin	553.3	
strophanthidol		443.2
proscillaridin A	429.1	

The LC-MS method was validated as follows. The linearity was evaluated by analyzing three batches of standard curves of all 14 DLCs over the concentration range of 1.0-400 nM. Good linearity was observed over the quantification range when a linear regression was used with least-squares regression ($1/\chi^2$). The correlation coefficients (r) were greater than 0.9900 for all analytical batches, with a bias within ± 10%. The intra- and interassay accuracy and precision were determined at the LLOQ (1.0 nM), low (10 nM), medium (50 nM), and high (250 nM) QC levels by analyzing three separate analytical batches in five replicates for each concentration. The intra- and interassay precision was less than 10 and 11%, respectively, and the bias ranged from -4.8 to 9.0% and -9.2 to 11.1%, respectively. These results were within an acceptable range, and the analytical method proved to be reproducible in terms of intra- and interassay accuracy and precision.

Analysis. All of the data are expressed as the mean ± SD of three independent experiments after correction for the uptake in CHO-parent and HEK-parent cells. To determine K_m and V_{max} of the transporters, concentration-dependent uptake data were fitted to the Michaelis-Menten equation using Graphpad Prism software (version 5.02;

GraphPad Software Inc., San Diego, CA). DLCs were screened for inhibitory properties using one-way ANOVA followed by Dunnett's post-hoc multiple comparisons. The unpaired Student's *t* test was used to compare groups in the DLC uptake assays.

Inhibition curves were analyzed with a one-site binding model fitted to the equation y = bottom + (top – bottom) / (1 + 10 $^{(\log IC50 - x).\ Hill\ slope}$) using GraphPad Prism. In this equation, x and y indicate log values of inhibitor concentration and uptake versus control, respectively.

Xcalibur software (Thermo Scientific, San Jose, CA, USA) was used to control the LC-MS system, and LCquan software (Thermo Scientific, San Jose, CA, USA) was used for sample data analysis.

The physicochemical properties of DLCs such as octanol: water partition coefficient (clogP) and polar surface area (tPSA) were calculated using the ChemBioOffice software package (CambridgeSoft, Cambridge, MA, USA), and H-bond donors and acceptors were obtained from the PubChem database (National Center for Biotechnology Information, Bethesda, MD, USA).

Results

Kinetic Characterization of NTCP, OATP1B1, and OATP1B3. Previously, CHO-NTCP, HEK-OATP1B1, and HEK-OATP1B3 cell lines were characterized at the functional and/or protein expression levels (Greupink *et al.*, 2011; Bosgra *et al.*, 2013; van de Steeg *et al.*, 2013). In the present study, to characterize the function of the transporters in the cell lines, the time- and concentration-dependent uptake of TCA by NTCP and $E_2$17βG by OATP1B1 and OATP1B3 was measured (Figure 2). Uptake of TCA by CHO-NTCP and CHO-P was measured in the presence of 1 μM TCA. NTCP-mediated uptake of TCA was linear over 4 min (Figure 2A). Concentration-dependent uptake of TCA by NTCP, measured after 1 min of incubation, was characterized by an apparent affinity (K_m) of 5.4 ± 1.8 μM and a maximum rate (V_{max}) of 229 ± 24 pmol/mg protein/min (Figure 2B), and this was comparable to the values previously shown by others (Hagenbuch & Meier, 1994; Meier *et al.*, 1997; Kim *et al.*, 1999; Greupink *et al.*, 2011). Time-dependent uptake of 2 μM $E_2$17βG was measured in HEK-P and HEK-OATP1B1 cells (Figure 2C). Because the uptake activity of HEK-OATP1B1 was linear over a time period of 4 min, an incubation time of 2 min was selected to study the kinetic characteristics of $E_2$17βG uptake by OAPTP1B1. The K_m and V_{max} values of $E_2$17βG for OATP1B1 were 10 ± 3.4 μM and 151 ± 14 pmol/mg protein/min, respectively. The kinetics of $E_2$17βG uptake by OATP1B1 was in accordance with the literature (Hsiang *et al.*, 1999; Tamai *et al.*, 2001; Hirano *et al.*, 2004). Uptake of $E_2$17βG (2 μM) in HEK-P

and HEK-OATP1B3 was also linear over the first 4 min. K_m and V_{max} of OATP1B3-mediated $E_2$17βG uptake measured at 3 min were 31 ± 8.5 μM and 67 ± 6.8 pmol/mg protein/min, respectively (Figures 2E, F), which is also in line with previously reported values (Hirano *et al.*, 2004).

Effect of DLCs on NTCP-Mediated Uptake of TCA and OATP1B1- and OATP1B3-Mediated Uptake of $E_2$17βG. To investigate the influence of DLCs on transporter activity, uptake rates of TCA by NTCP and of $E_2$17βG by OATP1B1 and OATP1B3 were measured in the absence and presence of 14 DLCs. We can exclude a cell toxicity effect, as we did not observe a DLC inhibitory pattern that was similar in all three cell lines. Figure 3A represents the effect of 10 and 100 μM of the individual DLCs on NTCP-mediated TCA uptake. The uptake of TCA by NTCP in the absence of DLCs was set at 100% (Ctrl: 48 ± 7 pmol/mg protein/min). The most potent inhibitor, proscillaridin A, inhibited transport activity by 77% at 100 μM. Digoxin inhibited uptake by 25 and 46% at concentrations of 10 and 100 μM, respectively. Convallatoxin, digitoxin, and ouabain (100 μM) inhibited TCA uptake by approximately 20%. Digoxigenin and gitoxigenin stimulated NTCP-mediated TCA uptake at 100 μM. Digoxin inhibited uptake by 25 and 46% at concentrations of 10 and 100 μM, respectively. Convallatoxin, digitoxin, and ouabain (100 μM) inhibited TCA uptake by approximately 20%. Digoxigenin and gitoxigenin stimulated NTCP-mediated TCA uptake at 10 μM by 32 and 43%, respectively, whereas they hardly affected transporter activity at 100 μM. TCA itself (100 μM unlabeled) inhibited NTCP by 90%.

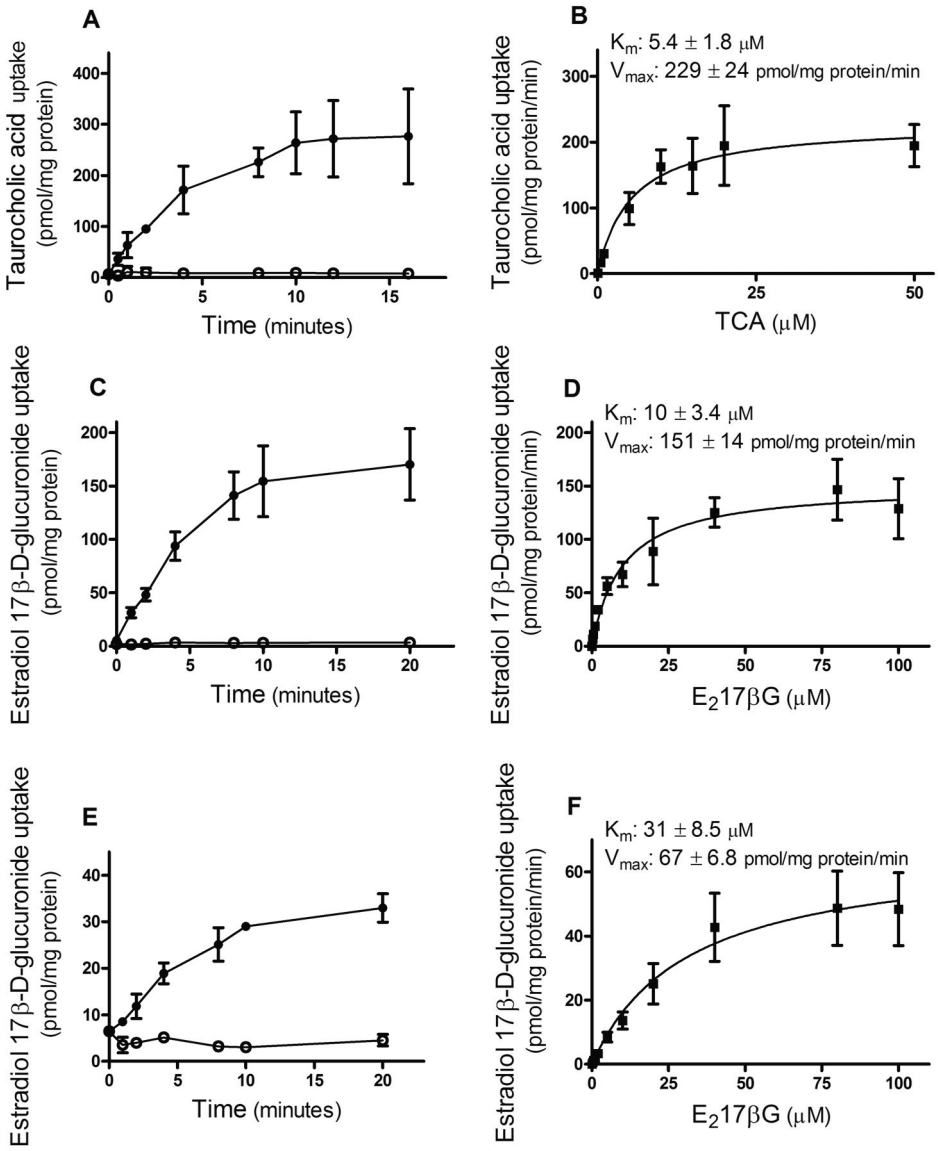


Figure 2. Time- and concentration-dependent transport of taurocholic acid and estradiol 17-β-D glucuronide in CHO-NTCP (A, B), HEK-OATP1B1 (C, D), and HEK-OATP1B3 (E, F), respectively. The NTCP-mediated transport of taurocholic acid (TCA) in CHO-NTCP was measured in the presence of 1 μM TCA (A) and at increasing concentrations of TCA (B) during 1 min incubation. Time course of the transport of 17-β-D-glucuronide ($E_2$17βG) by OAP1B1 (C) and OATP1B3 (E) in HEK cells was measured in the presence of 2 μM $E_2$17βG. The concentration-dependent transport of $E_2$17βG by OAP1B1 (D) and OATP1B3 (F) was measured during 2 and 3 min incubation times, respectively. CHO-P and HEK-P were used as controls (empty circles in A, C, and E), and their values were subtracted in panels B, D, and F. Data points represent the mean ± SD of experiments performed in triplicate.

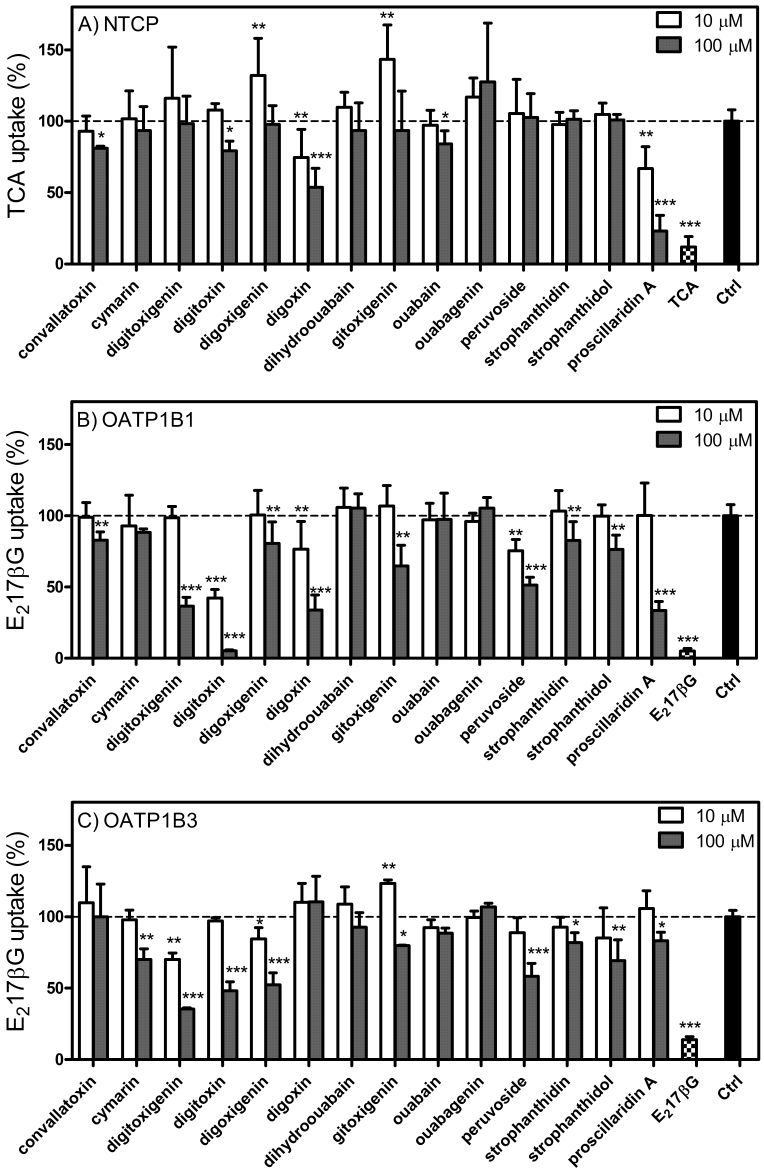


Figure 3. Effect of DLCs on TCA uptake by NTCP (A) and $E_2$17βG uptake by OATP1B1 (B) and OATP1B3 (C). Uptake was measured during 1 min at a concentration of 1 μM TCA for NTCP (A), during 2 min at a concentration of 2 μM $E_2$17βG for OATP1B1, and during 3 min at a concentration of 2 μM $E_2$17βG for OATP1B3. For all three transporters, uptake was measured in the absence (Ctrl) or presence of 14 DLCs at 10 and 100 μM. NTCP-, OATP1B1-, and OATP1B3-mediated uptake in the absence of DLCs (Ctrl) was set at 100% and was also measured in the presence of 100 μM of the corresponding unlabeled substrate. The mean ± SD values of three independent experiments are shown, and statistically significant differences are indicated (*, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$).

The uptake activity of OATP1B1 in the absence of DLCs was set at 100% (Ctrl: 57.8 ± 20 pmol/mg protein/min) (Figure 3B). Digitoxin, the most potent inhibitor of OATP1B1, inhibited uptake of $E_2$17βG by 95% at 100 μM. In addition, 100 μM digitoxigenin, digoxin, and proscillaridin A inhibited OATP1B1 by 58 and 66%, respectively. The inhibitory effect of 100 μM convallatoxin, digoxigenin, gitoxigenin, peruvoside, strophanthidin, and strophanthidol was less than 50%. The inhibitory potency of DLCs on OATP1B1-mediated transport can be summarized as digitoxin > digoxin = proscillaridin A > digitoxigenin > peruvoside > gitoxigenin. OATP1B3-mediated $E_2$17βG uptake was 17.2 ± 4.5 pmol/mg protein/min (Ctrl: 100%) (Figure 3C). The most potent inhibitor of OATP1B3, digitoxigenin, inhibited uptake by 65% at 100 μM. Digitoxin and digoxigenin inhibited uptake up to 50% at 100 μM, but most of the tested DLCs, such as cymarin, gitoxigenin, strophanthidin, strophanthidol, and proscillaridin A, inhibited OATP1B3 by less than 30%. Gitoxigenin stimulated OATP1B3 activity by 23% at 10 μM and inhibited transport by 20% at 100 μM. At the tested concentrations, DLCs were less potent inhibitors of OATP1B3 than OATP1B1.

Concentration-Dependent Inhibition of NTCP, OATP1B1, and OATP1B3 by DLCs. To analyze the inhibitory potency of DLCs against NTCP, OATP1B1, and OATP1B3 activity, DLCs with an inhibitory efficacy of 50% or more at 100 μM were selected for further study. The uptake rates of TCA by NTCP and $E_2$17βG by OATP1B1 and OATP1B3, expressed as the percentage of control, were plotted against the log value of increasing DLC concentrations (Figure 4). Proscillaridin A, the most potent NTCP inhibitor, exhibited an IC_{50} value of 22.0 ± 4.0 μM. Inhibitory potency against OATP1B1 could be ranked as follows: digitoxin (14.2 ± 0.3 μM) > proscillaridin A (47.0 ± 13.0 μM) > digoxin (63.0 ± 15.7 μM) > digitoxigenin (73.5 ± 16.2 μM) > peruvoside (90.3 ± 6.8 μM). Digitoxigenin was the most potent inhibitor of OATP1B3 (36.0 ± 12.8 μM), whereas the inhibitory potencies of digitoxin and digoxigenin were 3 times lower (129.0 ± 53.0 and 130.0 ± 54.4 μM, respectively).

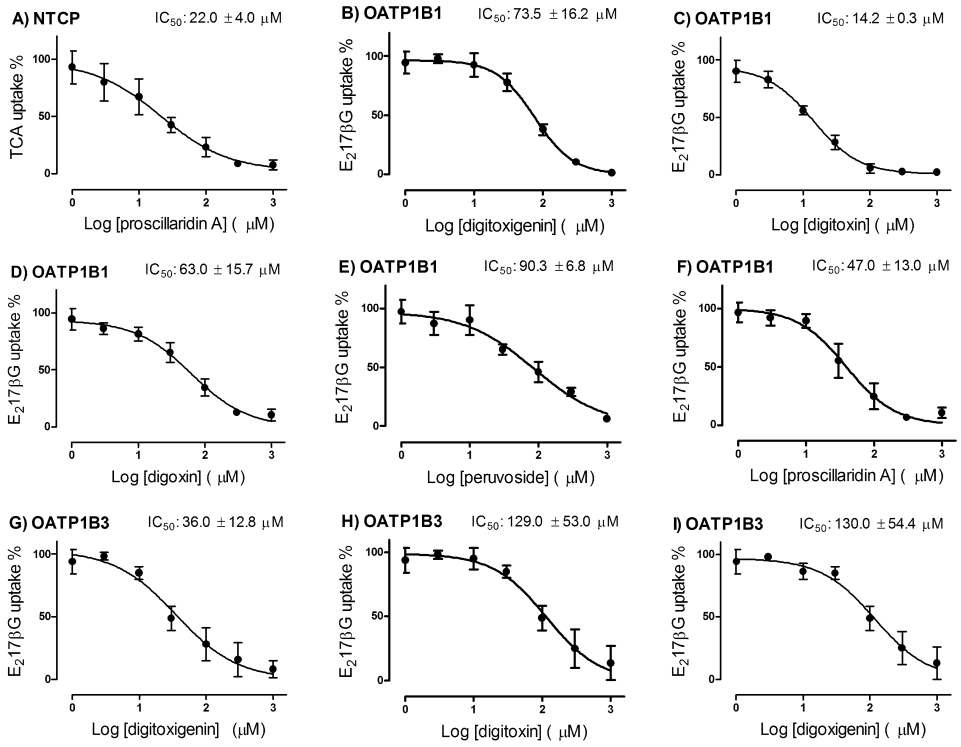


Figure 4. Concentration-dependent inhibition of NTCP (A), OATP1B1 (B-F), and OATP1B3 (G-I) by selected DLCs. CHO-P and CHO-NTCP were incubated with 1 μM TCA in the absence and presence of increasing concentrations of proscillaridin (A) for 1 min. $E_2$17βG (2 μM) was incubated with HEK-P and HEK-OATP1B1 in the presence of increasing concentrations (1-1000 μM) of digitoxigenin (B), digitoxin (C), digoxin (D), peruvoside (E), and proscillaridin A (F) for 2 min and with HEK-P and HEK-OATP1B3 in the presence of increasing concentrations of digitoxigenin (G), digitoxin (H), and digoxigenin (I) for 3 min. NTCP-mediated uptake of TCA and OATP1B1- and OATP1B3-mediated uptake of $E_2$17βG in the absence of DLCs was 48 ± 6, 50 ± 10.6, and 19 ± 8 pmol/mg protein/min, respectively, which were set at 100%. The mean ± SD of three independent experiments is shown.

Identification of NTCP, OATP1B1, and OATP1B3 Substrates. Absolute uptake of DLCs (10 μM) after 1 h in parent and transporter-expressing cell lines was measured using LC-MS. Significant differences in DLC uptake could not be detected when comparing CHO-NTCP and CHO-parent cell lines (Figure 5 and Table 2). Also, no significant differences in DLC uptake could be detected when comparing HEK-OATP1B1 and HEK-parent cell lines (Figure 6 and Table 2). OATP1B3 showed statistically significant uptake of convallatoxin (230 ± 40% compared to control cells, $p < 0.001$), dihydroouabain (381 ± 14% of control, $p < 0.001$), ouabain (1140 ± 210% of control, $p < 0.001$), and ouabagenin (212 ± 10% of control, $p < 0.001$) (Figure 7 and Table 2). The OATP1B3 inhibitor rifampicin (Cui *et al.*, 2001) reduced the uptake of $E_2$17βG, convallatoxin,

dihydroouabain, ouabain, and ouabagenin in HEK-OATP1B3 cells, whereas it had no effect on the uptake of these compounds in HEK-parent cells (Figure 8). Transport activity of OATP1B3 and its inhibition by rifampicin was confirmed by using $E_2$17βG as the probe substrate in our assay (Figure 8E).

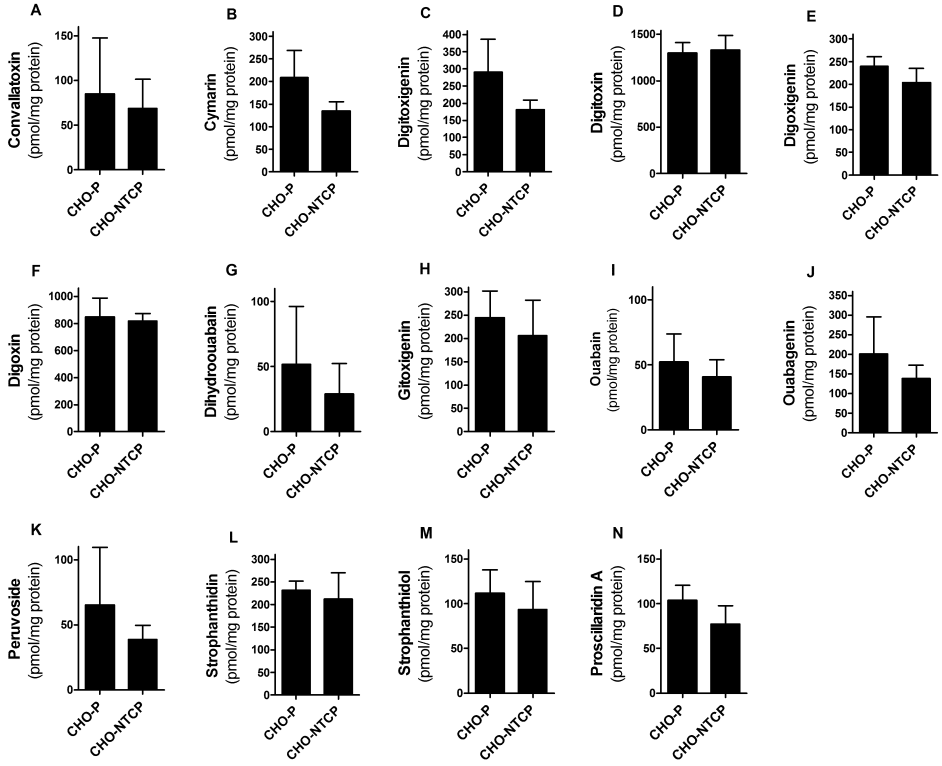


Figure 5. Uptake of DLCs in CHO-P and CHO-NTCP. Uptake of 14 DLCs was measured by LC-MS after a 1 h incubation of CHO-P and CHO-NTCP with convallatoxin (A), cymarin (B), digitoxigenin (C), digitoxin (D), digoxigenin (E), digoxin (F), dihydroouabain (G), gitoxigenin (H), ouabain (I), ouabagenin (J), peruvoside (K), strophanthidin (L), strophanthidol (M), and proscillaridin A (N). Each condition was performed in triplicate, and the mean ± SD of three independent experiments is shown.

Discussion

To understand the variation in the pharmacokinetic profile of different DLCs, we studied the interaction of 14 DLCs with liver uptake transporters NTCP, OATP1B1, and OATP1B3. Whereas we found little interaction with NTCP, several DLCs inhibited OATP1B1 and OATP1B3 transport activity. Moreover, OATP1B3 transported four DLCs.

Table 2. Accumulation of DLCs in CHO-P, CHO-NTCP, HEK-P, and HEK-OATPs

Compounds	Accumulation in NTCP vs. control (%)	Accumulation in OATP1B1 vs. control (%)	Accumulation in OATP1B3 vs. control (%)
convallatoxin	75 ± 46 (0.75)	87 ± 16 (0.9)	231 ± 93 (2.3) [a]
cymarin	66 ± 17 (0.7)	123 ± 12 (1.2)	115 ± 13.5 (1.2)
digitoxigenin	66 ± 18 (0.7)	92 ± 20 (0.9)	107 ± 26 (1.1)
digitoxin	102 ± 12 (1.0)	56 ± 7.7 (0.6)	95 ± 13 (0.95)
digoxigenin	86 ± 18 (0.9)	104 ± 14 (1.0)	113 ± 9.7 (1.1)
digoxin	96 ± 6.6 (1.0)	129 ± 23 (1.3)	116 ± 14 (1.2)
dihydroouabain	62 ± 7.3 (0.6)	79 ± 18 (0.8)	381 ± 35 (3.8) [a]
gitoxigenin	82 ± 14.5 (0.8)	84 ± 5.3 (0.8)	102 ± 18 (1.0)
ouabain	83 ± 17 (0.8)	93 ± 38 (0.9)	1139 ± 503 (11.4) [a]
ouabagenin	76 ± 22 (0.8)	112 ± 20 (1.1)	212 ± 23 (2.1) [a]
peruvoside	75 ± 33 (0.75)	107 ± 14 (1.1)	157 ± 13 (1.6)
strophanthidin	91 ± 23 (0.9)	117 ± 6.2 (1.2)	104 ± 14 (1.0)
strophanthidol	82 ± 23 (0.8)	107 ± 6.7 (1.1)	113 ± 26 (1.1)
proscillaridin A	75 ± 20 (0.75)	100 ± 17 (1.0)	130 ± 11 (1.3)

The accumulation of each DLC (10 μM) in CHO-P and HEK-P during a 1 h incubation was set at 100% (control), and the mean ± SD values of three independent experiments measuring the accumulation of DLCs in CHO-NTCP and HEK-OATPs were compared to that of the control using a one-way ANOVA test. [a] $p < 0.001$.

Looking more closely at the interactions of DLCs with NTCP, the presence of a sugar moiety appears to improve inhibition of NTCP activity (based on the comparison of sugar-conjugated DLCs digitoxin, digoxin, ouabain, and convallatoxin to that of unconjugated congeners digitoxigenin, digoxigenin, ouabagenin, and strophanthidin). This effect was most clearly observed for the digoxin/digoxigenin pair, whereas it was less prominent for other glycone/aglycone pairs. Furthermore, a hydroxyl group at position 12 (digoxin) increases the inhibitory efficacy compared to digitoxin (no hydroxyl group in the 12 position). Among the 14 tested DLCs, proscillaridin A was the most potent inhibitor of NTCP. Proscillaridin A's IC_{50} (22 μM) exceeded plasma concentrations reported in the clinic (systemic concentration, 1 nM; portal vein concentration, 3.5 nM) (Andersson *et al.*, 1977a), so no clinically relevant inhibitory effect on bile salt transport is expected for this compound. Although NTCP is known to be capable of transporting drug-like molecules, none of the tested DLCs was an NTPC substrate; hence, DDIs on the level of NTCP with DLCs as a victim drug are unlikely to occur.

OATP1B1-mediated uptake of $E_2$17βG was inhibited by digitoxin, digoxin, proscillaridin A, digitoxigenin, peruvoside, and gitoxigenin, whereas convallatoxin, cymarin, digoxigenin, strophanthidin, and strophanthidol hardly inhibited transporter activity. Inhibition of OATP1B1 by digoxin was also reported in previous studies (Badolo *et al.*, 2010; Karlgren *et al.*, 2012a; Karlgren *et al.*, 2012b). Badolo *et al*. showed that 20 μM digoxin inhibited $E_2$17βG uptake in hepatocytes by 56% and in HEKO-ATP1B1 cells by 36% (Badolo *et al.*, 2010), which is in line with our study. Karlgren *et al*. reported a 50% reduction of OATP1B1 activity in HEK293 by 20 μM ouabain (Karlgren *et al.*, 2012a). However, we did not observe any inhibition of OATP1B1 by ouabain (10 and 100 μM).

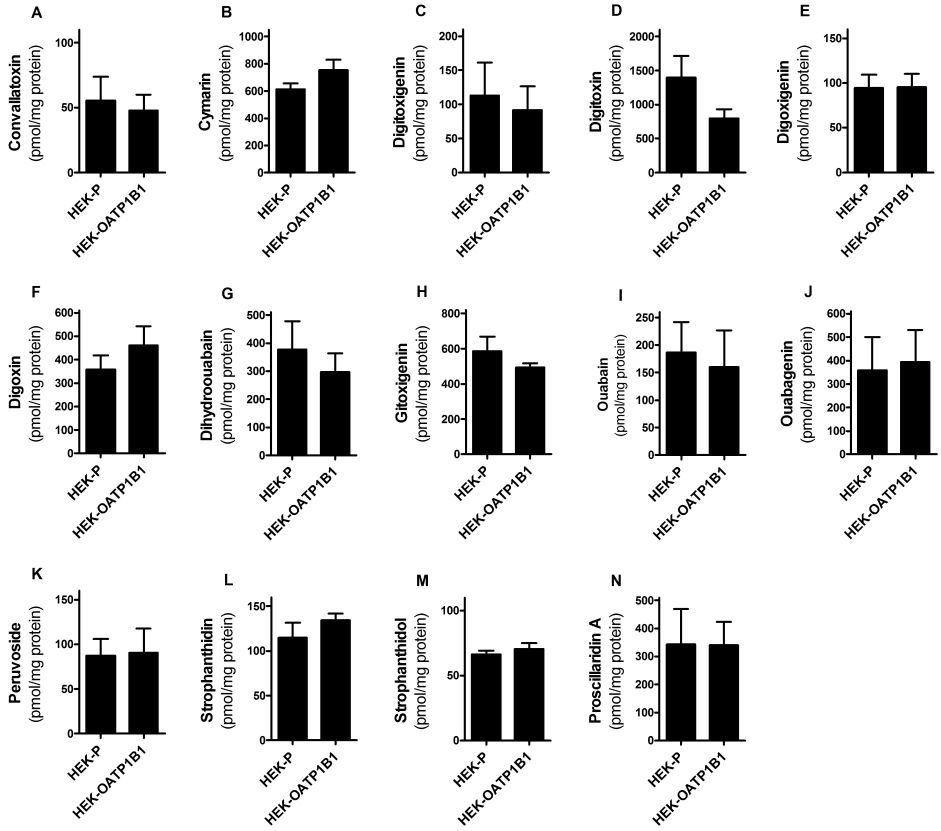


Figure 6. Uptake of DLCs in HEK-P and HEK-OATP1B1. The cell lines were incubated with convallatoxin (A), cymarin (B), digitoxigenin (C), digitoxin (D), digoxigenin (E), digoxin (F), dihydroouabain (G), gitoxigenin (H), ouabain (I), ouabagenin (J), peruvoside (K), strophanthidin (L), strophanthidol (M), and proscillaridin A (N) for 1 h, and uptake was measured by LC-MS. Each condition was performed in triplicate, and the mean ± SD of three independent experiments is shown.

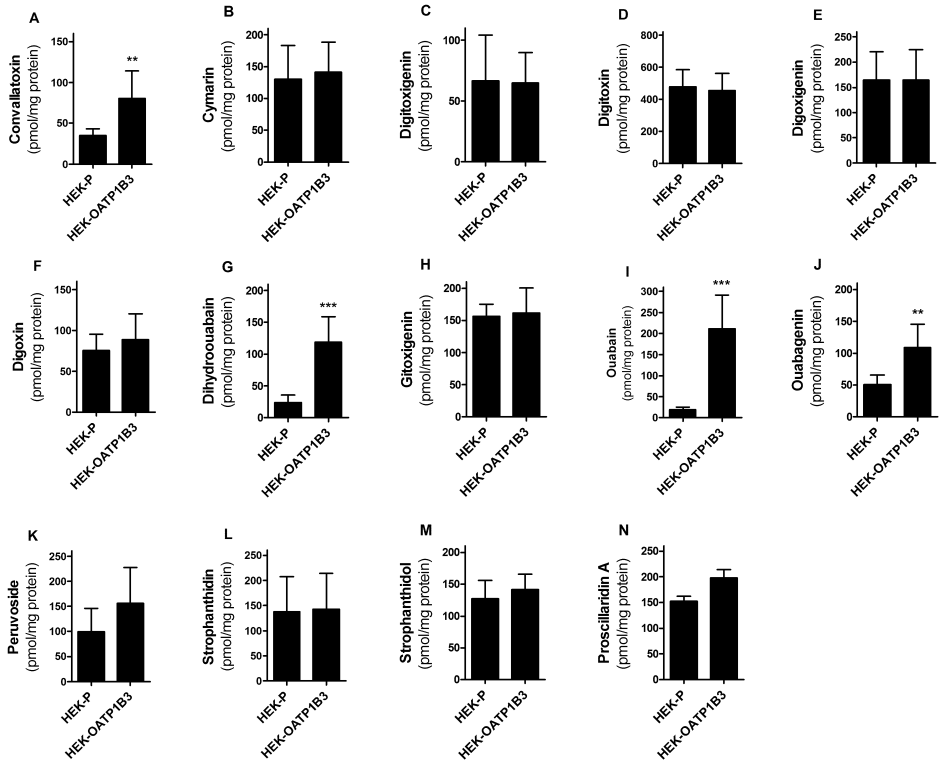


Figure 7. Uptake of DLCs in HEK-P and HEK-OATP1B3. Uptake of convallatoxin (A), cymarin (B), digitoxigenin (C), digitoxin (D), digoxigenin (E), digoxin (F), dihydroouabain (G), gitoxigenin (H), ouabain (I), ouabagenin (J), peruvoside (K), strophanthidin (L), strophanthidol (M), and proscillaridin A (N) in HEK-P and HEK-OATP1B1 was measured after a 1 h incubation by LC-MS. Each condition was performed in triplicate, and the mean ± SD of three independent experiments is shown. An unpaired Student's *t* test was used to compare DLC uptake in HEK-OATP1B1 versus HEK-P (**, $p < 0.01$; ***, $p < 0.001$).

Reported plasma concentrations for digoxin and digitoxin are 3 and 20 nM, respectively (portal vein concentration of 1.5 nM for digoxin (Andersson *et al.*, 1975)), whereas we found their IC_{50} values to be ~1000-fold higher. The chance of these compounds causing a relevant inhibition of OATP1B1-mediated drug transport is therefore very small and is, in fact, also in line with the absence of clinical reports of such an event (Shapiro *et al.*, 1970; Reitbrock & Woodcock, 1989). Whether the inhibitory potencies determined for the other DLCs against OATP1B1 are clinically relevant depends on the therapeutic plasma concentration windows for these compounds. To date, such clinical efficacy and safety data have not been reported to our knowledge.

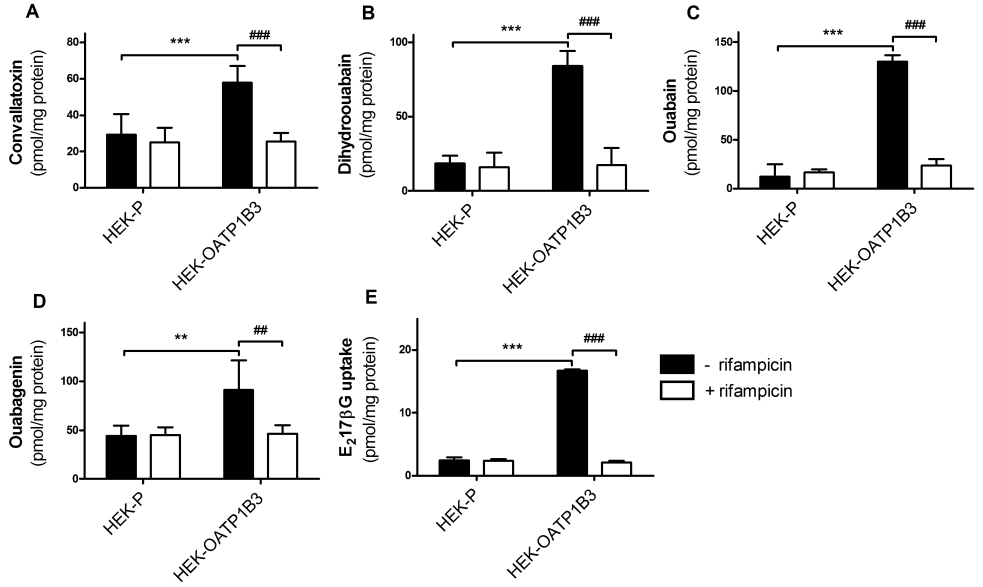


Figure 8. Inhibition of OATP1B3-mediated DLC transport. Uptake of DLCs was determined after incubation of 10 μM convallatoxin (A), dihydroouabain (B), ouabain (C), and ouabagenin (D) with HEK-P and HEK-OATP1B3 for 1 h in the absence (black bars) and presence (white bars) of rifampicin (100 μM). Uptake of $E_2$17βG (2 μM) in HEK-P and HEK-OATP1B3 was measured in the absence and presence of rifampicin (100 μM) after a 3 min incubation. The mean ± SD value of three independent experiments is shown. Uptake in HEK-OATP1B3 was compared to HEK-P (***, $p < 0.001$) and to the condition without rifampicin (###, $p < 0.001$) using an unpaired Student's *t* test.

In addition, the comparison of the inhibitory potencies against OATP1B1 by different DLCs also revealed structural features critical for interaction with the transporter. Digitoxin and digitoxigenin appeared to be more potent inhibitors than digoxin and digoxigenin, respectively, which demonstrated that the hydroxyl group at position 12 decreases inhibitory potency. Digoxin and digitoxin carry a sugar moiety at position 3, and they are better inhibitors of OATP1B1 than digoxigenin and digitoxigenin, which have a hydroxyl group at this position. This shows that the presence of a sugar moiety at position 3 could enhance the inhibitory effect of DLCs on OATP1B1 activity. This effect was not observed for the weak inhibitors, convallatoxin, and strophanthidin. Moreover, convallatoxin and cymarin have identical steroid bodies and slightly different sugar moieties; still, they share a low inhibitory potency against OATP1B1. In addition, the presence of a hydroxyl group at position 16 seems to lower the inhibitory potency of DLCs (gitoxigenin vs digitoxigenin).

Among the tested DLCs, digitoxigenin, digitoxin, and digoxigenin were the most potent inhibitors of OATP1B3. We show that digoxin and ouabain hardly influence the

transport activity of OATP1B3; however, Baldes *et al*. have shown that ouabain and digoxin inhibited OATP1B3-mediated Fluo-3 transport (Baldes *et al.*, 2006). Because the inhibitory effect of inhibitors on a transporter could be substrate-dependent (Tamai *et al.*, 2001; Noe *et al.*, 2007; Seithel *et al.*, 2007), the different OATP1B3 substrates ($E_2$17βG vs Fluo-3) could provide an explanation for the differing results. Moreover, Karlgren *et al*. reported 20 μM ouabain to be an inhibitor of OATP1B3-mediated 1 μM $E_2$17βG uptake (with 20% inhibition), whereas 20 μM digoxin did not inhibit 2 μM $E_2$17βG uptake by OATP1B3 in the present study, which is possibly related to the different substrate concentration used (Karlgren *et al.*, 2012b).

The absence of a sugar moiety at position 3 of DLCs appeared to improve binding to OATP1B3, because digitoxigenin, digoxigenin, and strophanthidin are better inhibitors than digitoxin, digoxin, and convallatoxin. Moreover, a cymarose sugar seems to be more favorable for binding than a rhamnose sugar (cymarin vs convallatoxin). In addition, the hydroxyl group at position 12 decreases the inhibitory potency (digoxin and digoxigenin vs digitoxin and digitoxigenin). Comparing gitoxigenin with digitoxigenin indicates that a hydroxyl group at position 16 reduces the inhibitory potency. Comparison of strophanthidin and strophanthidol indicates that a hydroxyl group at position 19 is better than a ketone for DLC binding.

It was shown previously that molecular weight, lipophilicity, polar surface, and presence of hydrogen-bond acceptors are the key factors for OATP1B1 and OATP1B3 inhibition (Gui *et al.*, 2009; Badolo *et al.*, 2010; Karlgren *et al.*, 2012a; Karlgren *et al.*, 2012b; Soars *et al.*, 2012; De Bruyn *et al.*, 2013). In previous studies, we found a similar correlation between lipophilicity and inhibitory potency of various statins against NTCP (Greupink *et al.*, 2011). Here, we did not observe an association between the inhibitory potency of DLCs against OATP1B1 and OATP1B3 and their physicochemical properties (molecular weight, octanol: water partition coefficient (clogP), polar surface area (PSA), and number of H donors and acceptors. Although no statistically significant correlation was found, our findings did show a tendency of lipophilic DLCs to be better inhibitors of OATP1B1 and OATP1B3 (Table 3).

Although none of the DLCs was a transporter substrate, we demonstrated that convallatoxin, dihydroouabain, ouabain, and ouabagenin are substrates of OATP1B3. It is the first time that convallatoxin, dihydroouabain, and ouabagenin have been reported as the substrates of OATP1B3. In a previous study by Kullak-Ublick *et al*., digoxin and ouabain uptake by OATP1B3 in *X. laevis* oocytes was shown (Kullak-Ublick *et al.*, 2001), but Kimoto et al. and Taub *et al*. did not observe any digoxin transport by OATP1B3 (Kimoto *et al.*, 2011; Taub *et al.*, 2011). Convallatoxin has been shown as an inhibitor of digoxin uptake in Caco-2 cells (Cavet *et al.*, 1996), and its uptake by rat intestine seemed to be transporter-dependent (Lauterbach, 1968).

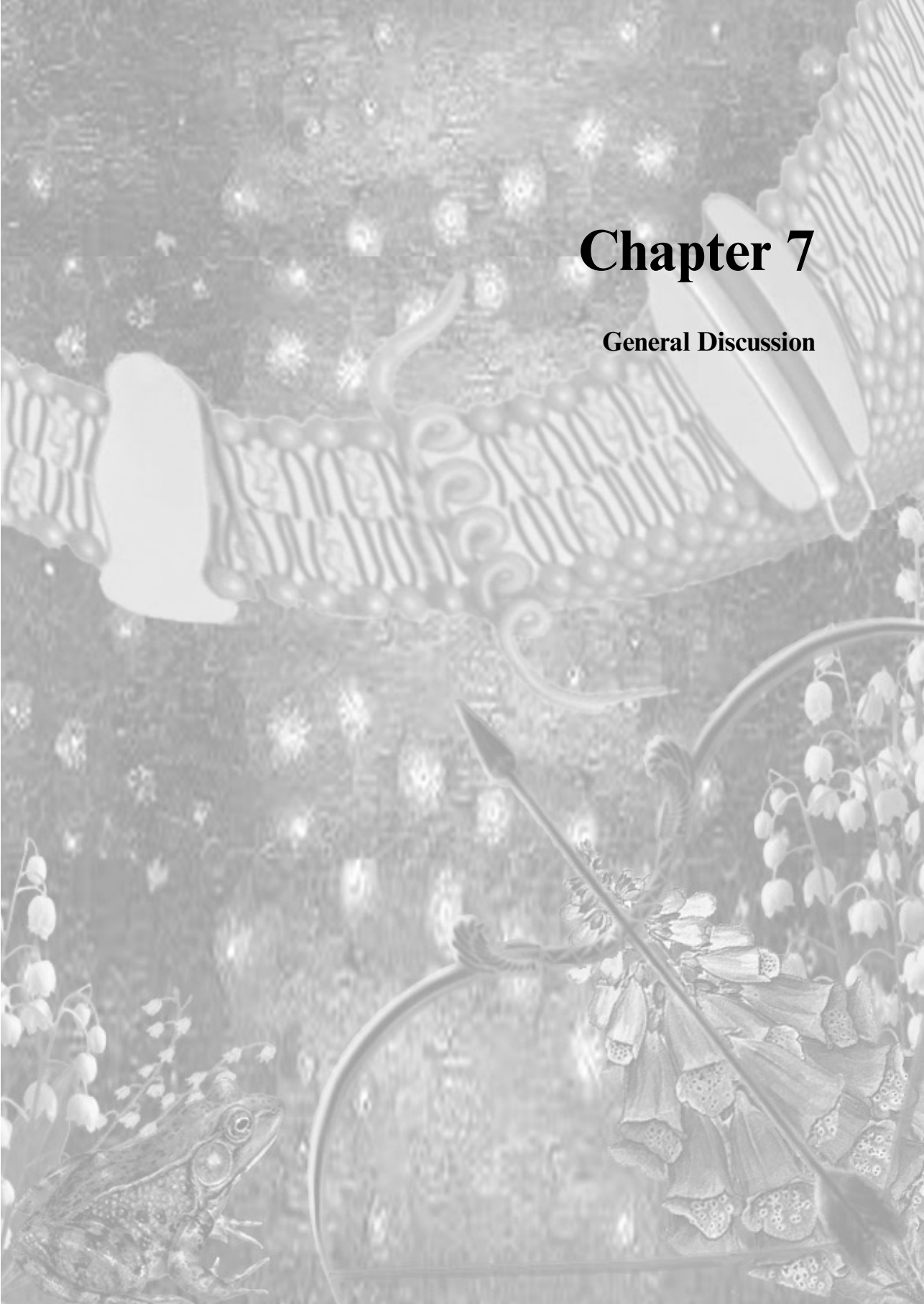
Table 3. Calculated physicochemical properties of DLCs

Compounds	ClogP [a]	tPSA [b]
convallatoxin	-0.67	162.98
cymarin	0.22	131.75
digitoxigenin	2.48	66.76
digitoxin	2.85	182.83
digoxigenin	1.05	86.99
digoxin	1.42	203.6
dihydroouabain	-1.8	206.6
gitoxigenin	2.25	86.99
ouabain	-1.66	206.6
ouabagenin	-1.32	147.68
peruvoside	0.32	131.75
strophanthidin	-0.3	104.06
strophanthidol	-0.28	107.22
proscillaridin A	2.55	125.68

[a] Calculated octanol: water partition coefficient.
[b] Calculated polar surface area.

The four DLCs that have been found as OATP1B3 substrates (convallatoxin, ouabain, dihydroouabain, and ouabagenin) do not inhibit the transporter under the conditions tested and are more hydrophilic than the other congeners (Table 2), which might be a characteristic of OATP1B3 substrates. Among newly found substrates of OATP1B3, ouabain (reported plasma concentration 0.7 nM) has been applied in therapy. Given the present data, ouabain pharmacokinetics may be affected by concomitant administration of potent OATP1B3 inhibitors (Selden & Smith, 1972). Such DDI have, however, not been reported. We are not aware of clinical studies investigating the potential for DDI of convallatoxin, dihydroouabain, and ouabagenin either.

In summary, we identified new DLC inhibitors of the liver uptake transporters, NTCP, OATP1B1, and OATP1B3. We showed that structural features such as the sugar moiety and hydroxyl groups play different roles in the interaction with these transporters. The sugar moiety decreases the interaction with OATP1B3, whereas it enhances the interaction with NTCP and OATP1B1. The hydroxyl group at position 12 enhances the interaction with NTCP, but it decreases the interaction with OATP1B1 and OATP1B3. In addition, we found that convallatoxin, dihydroouabain, ouabain, and ouabagenin are novel substrates of OATP1B3.



Chapter 7

General Discussion

Introduction

Digitalis-like compounds (DLCs) are ancient medications for heart failure, and are ranked in the top ten drugs leading to hospitalization due to their adverse effects. This is a result of their narrow therapeutic index, which is influenced at different pharmacokinetic levels such as absorption, metabolism, plasma-protein binding, and excretion.

Influx and efflux transport proteins play a key role in drug excretion and the consequent drug concentrations in plasma. Since the function of these transporters could be influenced by other drugs (substrates or inhibitors), drug-drug interaction (DDI) at the level of carrier-mediated DLC influx and efflux could occur. Therefore, a DLC that has a low interaction potential with influx and efflux transporters, would be less prone to DDI. In this study, different assays have been developed and used to study the interactions of DLCs with influx and efflux transporters. These assays were used to study DLCs for their properties as inhibitors and/or substrates of these transporters.

In this thesis, the interaction of DLCs with the ATP binding cassette (ABC) efflux transporters, multidrug resistance-associated proteins (MRP1, MRP2, MRP3 and MRP4), breast cancer resistant protein (BCRP), bile salt export pump (BSEP) and P-glycoprotein (P-gp) and the Solute Carrier (SLC) influx transporters, organic anion-transporting polypeptide (OATP1B1 and OATP1B3) and Na^+-taurocholate co-transporting polypeptide (NTCP) was studied (chapters 2-6). In addition, the amino acids that play a key role in the interaction of DLCs with P-gp were described in chapters 3 and 4. Here, the role of influx and efflux transporters to decrease DLC toxicity and prevent DDI was discussed. In addition, chemical substitutions that are important for the interaction of DLCs with the transporters are highlighted. Furthermore, after comparison of *in vitro* assays, key factors that should be considered in the selection of suitable assays to determine P-gp substrates were described.

Perspective of DLC toxicity management

The medical application of DLCs and their toxic effects were described by William Withering in "An account of the Foxglove, and some of its medical uses: with practical remarks on dropsy, and other diseases" in 1785 (Rahimtoola, 1975; Krikler, 1985; Rossner, 2006). Manifestations of DLC toxicity such as nausea, anorexia, abdominal pain, diarrhea, dizziness, headache, muscular weakness, visual complaints, psychic complaints and vomiting have been described when the plasma concentration of digoxin is above 4 nmol/L (3 μg/L) (Lely & van Enter, 1970; Rawlins, 1974; GAXS, 1988; Bauman *et al.*, 2006; Vivo *et al.*, 2008).

The therapeutic plasma concentration range of digoxin was reported between 1.0-2.5 nmol/L (0.8-2.0 μg/L) and concentrations above 2.5 nmol/L were considered toxic (Smith & Haber, 1970; Beller *et al.*, 1971). It has been shown that digoxin concentrations of 0.6–1.0 nmol/L (or 0.5–0.8 μg/L) were associated with a better cardiac outcome and less mortality (Adams *et al.*, 2002; Rathore *et al.*, 2003). Accordingly, a therapeutic digoxin concentration of 0.6–1.2 nmol/L (or 0.5–1.0 μg/L) was suggested for patients with heart failure. Currently, three clinical improvements have decreased digoxin and digitoxin toxicity: access to kits to monitor serum concentrations of digoxin and digitoxin (Duhme *et al.*, 1974), dosing guidelines using pharmacokinetic methods (Smith *et al.*, 1984; Vivo *et al.*, 2008), and the development of digoxin and digitoxin antibodies for toxicity treatment (Antman *et al.*, 1990).

In 1991, Beller *et al*. reported that more than 20% of the patients using digoxin showed probable to definite toxicity (Beller *et al.*, 1971). In the DIG (Digoxin Investigation Group) study in 1997, 12% toxicity was observed in patients using digoxin compared to 8% in the placebo group (DIG, 1997). It has been reported that 31% of patients, who were hospitalized because of heart failure, were prescribed with digoxin in 2001 in the United States. Although this percent has been decrease to 24% in 2004, showing a decline in digoxin usage, the number of toxic exposures to digoxin has not changed during this time period (Hussain *et al.*, 2006).

In modern era, digoxin toxicity still occurs because drug dosing fails frequently in patients with renal dysfunction and concomitant use of digoxin-interacting drugs (Gheorghiade *et al.*, 2006; Vivo *et al.*, 2008). Body weight, age, renal function, and concomitant medications are the key determinants that should be considered in digoxin dosing (Ehle *et al.*, 2011). The distribution volume of digoxin is large; therefore, patients with a lower lean body weight have less tissue for drug distribution and consequently need a lower dosage of digoxin (Gheorghiade *et al.*, 2004; Gheorghiade *et al.*, 2006). Furthermore, aging is often associated with a lower lean body weight and renal impairments that can influence digoxin distribution and excretion, respectively. The complete gastrointestinal absorption of digitoxin, its renal-independent excretion, and lower incidence of toxic side effects have ranked digitoxin less toxic than digoxin although the elimination half-life of digitoxin is longer than digoxin. In the United States, digoxin is still the most prescribed DLC (Roever *et al.*, 2000; Belz *et al.*, 2001), while in some countries such as Germany, Norway and France, digoxin has been replaced by digitoxin to decrease the incidence of digoxin toxicity. Although digitoxin is less toxic than digoxin, drug-drug interactions (DDIs) can occur for both cardiac glycosides. Several drugs such as diuretics, beta-blockers, antiarrhythmic agents, calcium channel blockers, and antibiotics increase the plasma concentration of digoxin and digitoxin and consequently their toxicity in the case of concomitant prescription (Steiness, 1978; Klein

et al., 1980; Bajaj *et al.*, 1991; Hanratty *et al.*, 2000; Belz *et al.*, 2001). In chapter 1 of this thesis, digoxin and digitoxin DDIs were extensively discussed.

After the identification of P-glycoprotein as digoxin and digitoxin transporter (de Lannoy & Silverman, 1992; Schinkel *et al.*, 1995; Pauli-Magnus *et al.*, 2001a), the underlying mechanism of DDIs at the transport level was revealed and it opened a new door to decrease these unwanted interactions. In conclusion, identification of DLCs transporters expands our knowledge of possible DDI sites and could be helpful to select DLCs that have similar therapeutic effects as digoxin and digitoxin, but are less prone to interactions and toxicity. To explore their therapeutic potential, we investigated potential interactions with DLCs at the level of influx and efflux transporters.

The role of efflux transporters in excretion of DLCs

The distribution and function of two transporter families, ATP-binding cassette efflux transporters (ABC) and solute-linked carriers (SLC) influx transporters play a key role in the absorption, distribution, metabolism, and excretion of drugs. These transporters are expressed in a many different organs with a barrier function, such as placenta, prostate, small intestine, brain, colon, liver, and kidney to protect against xenobiotics (Flens *et al.*, 1996; Maliepaard *et al.*, 2001; Doyle & Ross, 2003; Trauner & Boyer, 2003; Bart *et al.*, 2004; Fetsch *et al.*, 2006; Hilgendorf *et al.*, 2007; Huls *et al.*, 2008; Keppler, 2011; Garzel *et al.*, 2014). The induction or inhibition of these transporters is the underlying mechanism of drug-drug interactions and adverse drug effects.

In chapters 2, 3 and 4 of this thesis, the interaction of fourteen structurally related DLCs with efflux transporters BCRP, BSEP, MRP1, MRP2, MRP3, MRP4, and P-gp was described using membrane vesicles isolated from HEK293 cells overexpressing these transporters. We showed that digitoxin is the most potent inhibitor of BCRP-mediated estrone sulfate, BSEP-mediated taurocholic acid (TCA) and MRP4-mediated β-estradiol 17β-D-glucuronide ($E_2$17βG) transport. In addition, transport of $E_2$17βG by MRP1 and MRP3 was inhibited most potently by strophanthidol, whereas MRP2-mediated $E_2$17βG transport was not inhibited by DLCs, but its transport activity was stimulated by digitoxigenin more than 3-fold (chapter 2).

In chapter 3, we showed that P-gp-mediated N-methyl-quinidine (NMQ) transport was inhibited most potently by digitoxin and proscillaridin A. The comparison of chemical structures and inhibition of above-mentioned efflux transporters revealed the DLCs substitutions that influence their inhibitory potency (Figure 1). The presence of the sugar moiety at position C3 increased the inhibitory effect on BCRP, MRP1 and P-gp, whereas the presence of a hydroxyl group at this position increased the inhibitory

potency and the stimulatory effect on MRP4 and MRP2, respectively (chapter 2 and 3). In addition, the presence of the hydroxyl groups at the positions C12 and C16 reduced the inhibitory effect of DLCs on BCRP, BSEP, and P-gp, and interestingly, they also reduced the stimulatory effect on MRP2. The 12β hydroxyl group had a similar effect on MRP1 and MRP4, but this inhibitory and stimulatory effect was not observed for the 16β hydroxyl group. Moreover, the inhibitory potency of DLCs on BCRP, BSEP, MRP1, MRP3, and P-gp was reduced in the simultaneous presence of hydroxyl groups at positions C1 and C11. A hydroxyl group at position C19, however, increased the inhibitory potency (chapters 2 and 3). In chapters 2 and 3 of this thesis, we have shown that chemical substitutions at the positions C1, C3, C11, C12, C16, and C19 play a key role in the interaction of DLCs with the efflux transporters BCRP, BSEP, MRP1, MRP2, MRP3, MRP4, and P-gp (Figure 1). In addition, gitoxigenin is the DLC with the lowest interaction potential with efflux transporters (except stimulation of MRP2).

To determine whether DLCs are transported by BCRP, BSEP, MRP1, MRP2, MRP3, and MRP4, we have developed an indirect assay using the DLC affinity for Na, K-ATPase. The inhibitory effect on Na,K-ATPase has been used to demonstrate the presence of DLCs in different biological samples (Fishman, 1979; Whitmer *et al.*, 1982; Cloix *et al.*, 1984; Lichtstein *et al.*, 1999; De Pont *et al.*, 2009). In chapter 2, the extract of HEK293 membrane vesicles overexpressing BCRP, BSEP, MRP1, MRP2, MRP3 and MRP4 separated by filtration after incubation with DLCs in the absence and presence of ATP was used in a Na,K-ATPase-[^{3}H]ouabain replacement assay. Inhibition of [^{3}H] ouabain binding by the filtered membrane vesicles could demonstrate the transporter-mediated uptake of DLCs in these vesicles. Convallatoxin was accumulated in P-gp membrane vesicles confirming that convallatoxin is a P-gp substrate (further discussed in chapter 4), but we did not observe a significant accumulation of other DLCs in transporter-overexpressing membrane vesicles. High accumulation of digitoxigenin, digitoxin, and digoxin in control membrane vesicles implied that these lipophilic DLCs pass or enter the vesicle membrane by passive diffusion (chapter 2).

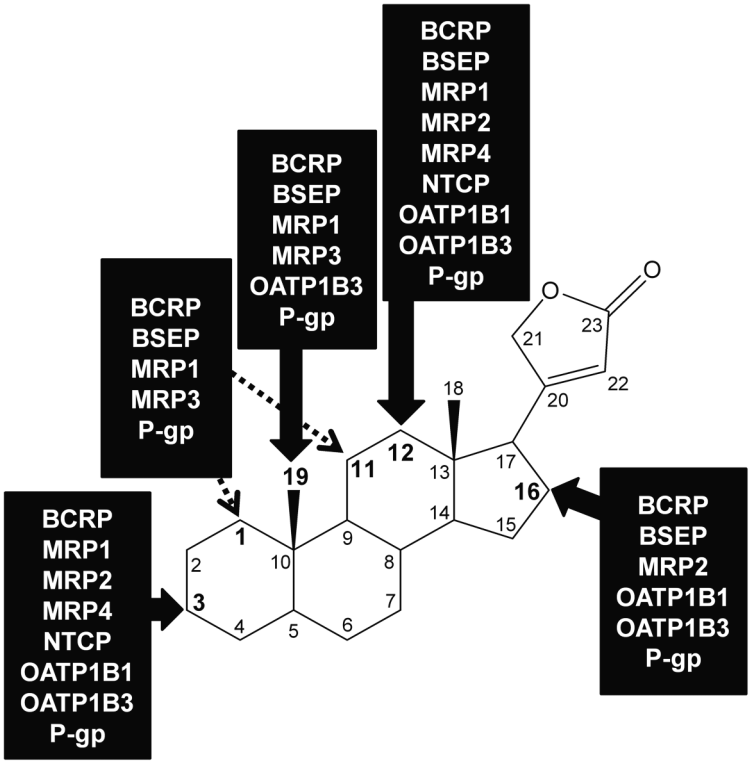


Figure 1. Interacting positions of cardenolides with influx and efflux transporters. The positions, C1, C3, C11, C12, C16, and C19 whose chemical substitutions (a hydroxyl group or a sugar moiety) influence the interaction of cardenolides with the influx (NTCP, OATP1B1, and OATP1B3) and the efflux (BCRP, BSEP, MRP1, MRP2, MRP3, MRP4, and P-gp) transporters are presented. Dashed arrows represent the simultaneous role of positions C1 and C11 of cardenolides.

In chapter 4, we investigated the transport of fourteen DLCs in membrane vesicles isolated from HEK293 cells overexpressing P-gp. We developed a liquid chromatography-mass spectrometry (LC-MS) method to quantify DLCs in the samples directly. We showed that convallatoxin was transported into these vesicles ATP-dependently and its transport was inhibited by the P-gp-inhibitor elacridar. It is the first time that convallatoxin, derived from *Convallaria majalis* (Lily of the valley), is reported as a P-gp substrate. An *in vivo* study in rats revealed that convallatoxin was highly accumulated in rat kidney cortex in presence of elacridar. As P-gp is expressed predominantly in renal proximal tubule cells, which are situated in the kidney cortex (Cordon-Cardo *et al.*, 1990), the high renal cortical accumulation in presence of elacridar underlined the P-gp involvement in the renal excretion of convallatoxin (chapter 4).

In addition, we studied the transport of structurally-related DLCs by P-gp in a cellular accumulation assay using a Madin-Darby canine kidney (MDCK) cell line constitutively

overexpressing human P-gp (MDCK-P-gp) (chapter 5). The cellular accumulation of DLCs in MDCK-P-gp was measured and compared with control MDCK using LC-MS. In contrast to the results obtained with the vesicular transport assay (chapter 4), digitoxin, digoxigenin, strophanthidin and proscillaridin A were transported by P-gp in the cellular accumulation assay (chapter 5) and their transport was significantly inhibited by elacridar. Since digitoxin and proscillaridin A accumulation in MDCK-P-gp was increased in the presence of the BCRP inhibitor Ko143 (Cooray *et al.*, 2002), these two DLCs might also be transported by endogenously expressed BCRP in the MDCK-P-gp cell line. P-gp-mediated transport of strophanthidin and proscillaridin A and their inhibition by elacridar was confirmed using the cellular accumulation assay in a conditionally immortalized proximal tubule epithelial cell line (ciPTEC) (chapter 5). In addition, BCRP-mediated proscillaridin A transport was also confirmed in the ciPTEC cellular accumulation assay. P-gp and BCRP have an overlapping function and a number of studies have indeed discussed the interaction of BCRP with digoxin. As it was shown by our *in vitro* studies (chapter 2), digoxin is an inhibitor of BCRP, however, it is not transported by BCRP (Table 1) (Pavek *et al.*, 2005; Huang *et al.*, 2012). In chapters 2, 3, 4, and 5 of this thesis, the DLCs interactions with the efflux transporters, BCRP, BSEP, MRP1, MRP2, MRP3, and MRP4 were investigated.

P-glycoprotein amino acid residues interacting with DLCs

To elucidate the role of amino acid residues in DLC-P-gp interactions, the effect of DLCs on the transport of NMQ by ten P-gp alanine mutants was investigated in a HEK293 membrane vesicular assay (chapter 3). We showed that Ile306, Phe343, Phe728, Thr945 and Leu975 are the key residues for P-gp-mediated transport of NMQ (Figure 2), which is in agreement with previous studies that showed the involvement of these amino acids in the interaction of P-gp with verapamil, vinblastine, cyclosporine A and colchicine (Aller *et al.*, 2009; Loo *et al.*, 2009). The conserved NMQ transport activity of P-gp mutants, L65A, F336A, I340A, F942A and V982A, exhibited that NMQ could be transported by P-gp lacking these residues, however, Leu65, Phe942 and Val982 do play a key role in verapamil transport (Loo *et al.*, 2006a; Aller *et al.*, 2009; Loo *et al.*, 2009).

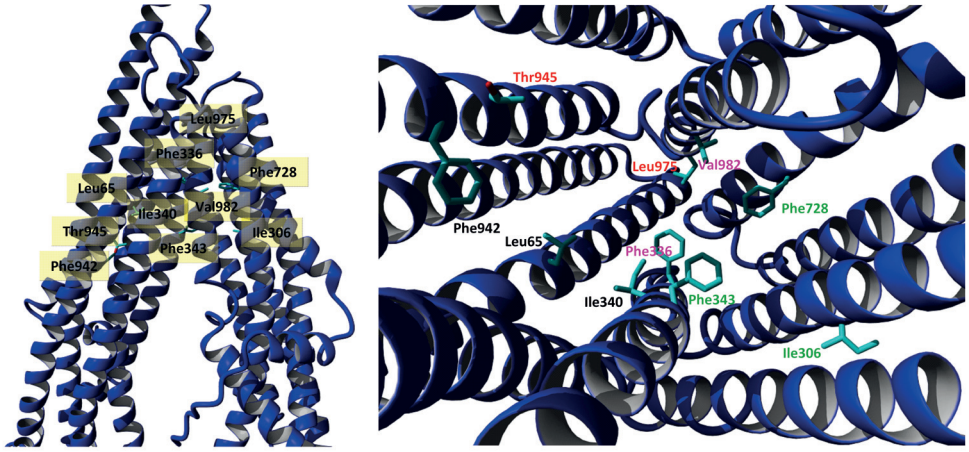


Figure 2. Positions of P-gp residues in the mutagenesis study. The positions of ten residues that were mutated to alanine are located in the P-gp transmembrane domains (left). The amino acids (Leu975 and Thr 945) that are important for NMQ transport activity of P-gp are shown in red and the key residues (Phe336 and Val982) for the convallatoxin transport are depicted in purple. The necessary residues (Ile306, Phe343, and Phe728) for transport of both NMQ and convallatoxin are shown in green.

The comparison of the inhibitory potency of structurally related DLCs underlined the importance of P-gp residues, Phe336 and Ile340, for interaction with the C3 and C12 positions of DLCs. It was also shown previously that the sugar moiety at position C3 influences the pharmacokinetic properties of DLCs (Thomas *et al.*, 1974; Smith *et al.*, 1984; Brown *et al.*, 1986).

In chapter 4 of this thesis, convallatoxin was introduced as a new substrate of P-gp in the vesicular transport assay and *in vivo*. Next, we investigated the role of P-gp residues in the transport of convallatoxin as a substrate. Interestingly, Ile306, Phe343, and Phe728, which were key residues for NMQ transport activity of P-gp, were also important for convallatoxin transport. However, the mutation of Phe343 to alanine altered the transport activity of P-gp for convallatoxin less than that for NMQ. Although, P-gp transport activity of NMQ was conserved after mutation of Val982 to alanine, it resulted in a reduction of P-gp-mediated convallatoxin transport by 50%. The involvement of Phe336 residue in the interaction of P-gp with DLCs, which was found in chapter 3, was also confirmed for convallatoxin in chapter 4. It was concluded that Phe336 and Ile340 interact with cymarin, digoxin, peruvoside, and proscillaridin A, and Phe336 and Val982 are necessary for P-gp-mediated transport of convallatoxin (Figure 2).

The role of hepatic influx transporters in DLCs uptake

In chapter 6, the interaction of DLCs with SLC transporters was studied. A cellular accumulation assay was used to study the effect of DLCs on OATP1B1 and OATP1B3-mediated E217βG uptake and NTCP-mediated TCA uptake. A number of DLCs could inhibit OATP1B1 and OATP1B3, but only proscillaridin A was a potent inhibitor of NTCP. The interaction of structurally-related DLCs with these transporters provided information about the chemical features of DLCs for inhibition of the uptake transporters. The sugar moiety at the C3 position increased the inhibitory potency against NTCP and OATP1B1, whereas inhibition of OATP1B3 was observed in the presence of a hydroxyl group at the C3 position. The presence of a hydroxyl group at the C12 position reduced inhibition of OATP1B1 and OATP1B3, but increased NTCP inhibition. Moreover, a hydroxyl group at C16 decreased the interaction of DLCs with OATP1B1 and OATP1B3. However, if a hydroxyl group was present at position C19 it increased the inhibitory potency against OATP1B3 (Figure 1).

Next, we showed that the hydrophilic DLCs, convallatoxin, dihydroouabain, ouabain, and ouabagenin were transported by OATP1B3 and their transport was inhibited in the presence of rifampicin (chapter 6). The OATP1B3-mediated uptake of digoxin and ouabain was also reported by Kullak-Ublick *et al.*, however, we and others did not observe digoxin as an OATP1B3 substrate (Kullak-Ublick *et al.*, 2001; Kimoto *et al.*, 2011; Taub *et al.*, 2011). Since the hydrophilic DLCs could not pass the plasma membrane by passive diffusion, the presence of an uptake transporter for the entrance of these compounds into excretory organs such as the liver is necessary. The lipophilic DLCs, digitoxin, digoxin, and digitoxigenin were highly taken up in membrane vesicles as was observed using ouabain-Na,K-ATPase replacement (chapter 2) and LC-MS quantification (chapter 4). This suggests that lipophilic DLCs do not need an uptake transporter to enter liver cells. In addition, the minor accumulation of convallatoxin and the absence of ouabain, dihydroouabain and ouabagenin accumulation in MDCK and ciPTEC cell lines (chapter 5) imply the requirement of an uptake transporter for these hydrophilic DLCs (Table 1).

Table 1. Interaction of DLCs with drug efflux and influx transporters.

DLCs	Efflux transporters							Influx transporters		
	BCRP	BSEP	MRP1	MRP2	MRP3	MRP4	P-gp	NTCP	OATP1B1	OATP1B3
Convallatoxin	Inhibitor [2]						Inhibitor [3]			
							Substrate [4] (vesicular transport)			Substrate [6]
							Substrate [4] (*in vivo*)			
Cymarin				Stimulator [2]			Inhibitor [3]			
Digitoxigenin	Inhibitor [2]			Stimulator [2]		Inhibitor [2]			Inhibitor [6]	Inhibitor [6]
Digitoxin	Inhibitor [2]	Inhibitor [2]	Inhibitor [2]	Stimulator [2]			Inhibitor [3]		Inhibitor [6]	Inhibitor [6]
							Substrate [5] (cellular accumulation *)			
							Substrate [A] (transwell transport [#])			
Digoxigenin			Stimulator [2]				Substrate [5] (cellular accumulation)			
							Substrate [B] (transwell transport)			
Digoxin	Inhibitor [2]						Inhibitor [3]		Inhibitor [6]	Inhibitor [6]
	Inhibitor [C]						Substrate [5] (ciPTEC accumulation)		Inhibitor [D]	Inhibitor [E]
							Substrate [F-G] (transwell transport)			Substrate [H]
							Substrate [I] (*in vivo*)			
Dihydroouabain										Substrate [6]
Gitoxigenin				Stimulator [2]						
Ouabagenin										Substrate [6]
Ouabain			Inhibitor [J]						Inhibitor [K]	Inhibitor [E]
										Substrate [6]
										Substrate [H]
Peruvoside	Inhibitor [2]	Inhibitor [2]	Inhibitor [2]	Stimulator [2]			Inhibitor [3]			
Strophanthidin	Inhibitor [2]		Stimulator [2]	Stimulator [2]	Inhibitor [2]		Substrate [5] (cellular accumulation)			
Strophanthidol	Inhibitor [2]		Inhibitor [2]		Inhibitor [2]		Inhibitor [3]			
Proscillaridin A							Inhibitor [3]	Inhibitor [6]	Inhibitor [6]	
							Substrate [5] (cellular accumulation)			

* Unpolarized cells overexpressing transporters such as MDCK-P-gp, [#] Polarized cells overexpressing transporters such as LLC-PK1 or MDCK-P-gp. [2-6] Referenced to chapters in this thesis, [A-K] References to literature: [A] (Pauli-Magnus *et al.*, 2001a), [B] (Hughes & Crowe, 2010), [C] (Pavek *et al.*, 2005), [D] (Badolo *et al.*, 2010), [E] (Baldes *et al.*, 2006), [F-G] (de Lannoy & Silverman, 1992; Pauli-Magnus *et al.*, 2001a), [H] (Kullak-Ublick *et al.*, 2001), [I] (Schinkel *et al.*, 1995), [J] (Valente *et al.*, 2007), [K] (Karlgren *et al.*, 2012b).

The vesicular transport assay versus the cellular accumulation assay

The vesicular transport assay has been used extensively to investigate the interaction of compounds with the carrier-mediated transport of a substrate (Leslie *et al.*, 2001; Hooiveld *et al.*, 2002; Breedveld *et al.*, 2004; El-Sheikh *et al.*, 2007; Pedersen *et al.*, 2008; Matsson *et al.*, 2009). In this thesis, the vesicular transport assay was used to study the interaction of DLCs with various efflux transporters (chapters 2 and 3), to identify P-gp substrates (chapter 4) and to analyze transport activity of P-gp mutants (chapters 3 and 4).

In the vesicle transport assay, digoxin, an established substrate of P-gp (de Lannoy & Silverman, 1992; Schinkel *et al.*, 1995), was not recognized as a P-gp substrate using [^{3}H] ouabain-Na,K-ATPase replacement and LC-MS methods to determine and quantify digoxin. Because of its relatively high lipophilicity digoxin likely diffuses passively into the membrane vesicles, leading to high background levels and a small difference between uptake into control and P-gp vesicles (Figure 3). The high uptake values of digoxin in control vesicles were also observed using [^{3}H]ouabain-Na,K-ATPase replacement and LC-MS methods (chapter 2 and 4). This confirms that the vesicular transport assay is not suitable for studying digoxin as a P-gp substrate. Only one study reports P-gp-mediated digoxin transport in a vesicular transport assay using trophoblast membrane vesicles isolated from human placenta (Ushigome *et al.*, 2003). Ushigome *et al*. showed that the uptake of digoxin by P-gp membrane vesicles is significantly higher in the presence of ATP than AMP, however, digoxin uptake by a control vehicle, which contains no P-gp expression, was not demonstrated.

The vesicular transport assay is often used to characterize the kinetics of a transporter by determining parameters such as V_{max}, K_m and IC_{50} (chapters 3 and 4) (Schlemmer & Sirotnak, 1994; Karlsson *et al.*, 2010; Elsby *et al.*, 2011; Heredi-Szabo *et al.*, 2013; Marchetti *et al.*, 2013). It is also a suitable model to study the effect of individual amino acid residues on the activity and inhibition of a transporter using mutagenesis studies (chapter 3) (Zelcer *et al.*, 2003; Schaefer *et al.*, 2006; El-Sheikh *et al.*, 2008; Wittgen *et al.*, 2012).

Efflux transporters protect cells from toxins and this function has been applied to develop an assay in which the cellular accumulation of DLCs was quantified using LC-MS to identify P-glycoprotein substrates (chapter 5). As this assay is based on the passive diffusion of test compounds into cells, it is appropriate for lipophilic compounds and not for hydrophilic membrane impermeable compounds. Also for highly lipophilic compounds it could be difficult to identify transporter involvement, because the rate of passive diffusion out of the cells can be higher than the rate of P-gp-mediated efflux or P-gp can be saturated by high intracellular concentration of these compounds (Figure 3) (Eytan *et al.*, 1996; Polli *et al.*, 2001).

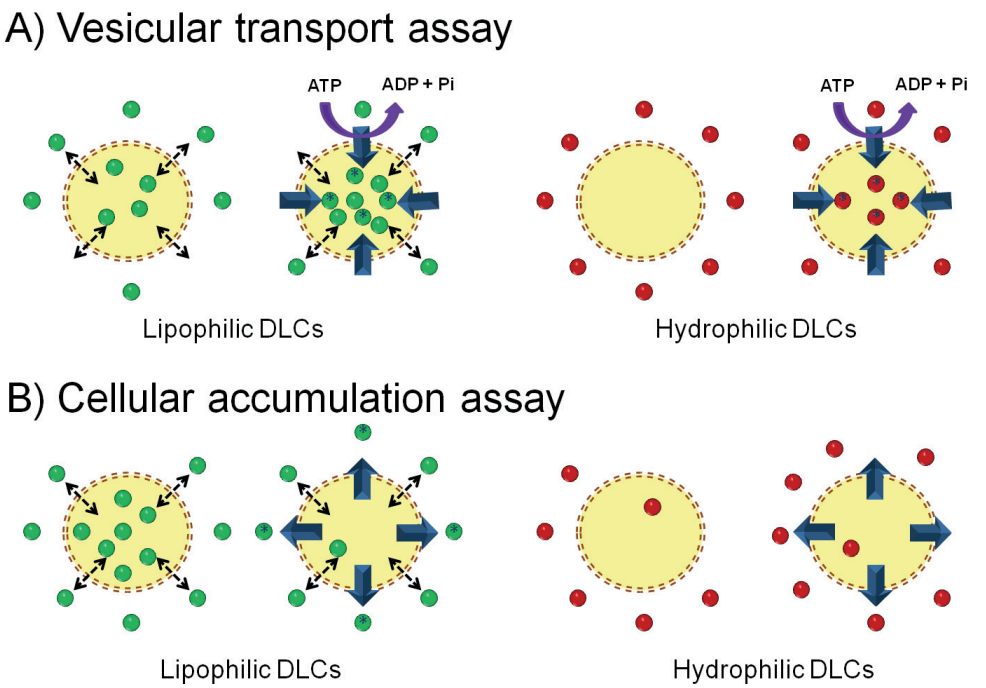


Figure 3. Behaviour of lipophilic and hydrophilic DLCs as transporter substrates in the vesicular transport (A) and the cellular accumulation (B) assays. Lipophilic and hydrophilic DLCs are depicted by green and red circles, respectively, and the DLCs that were effluxed by a transporter have been marked with black star. The blue and dashed arrows represent the efflux transporter and passive diffusion, respectively.

Furthermore, digoxin was not identified as a P-gp substrate in the MDCK cellular accumulation assay; however, it was in the ciPTEC accumulation assay. The expression of OATP4C1 in ciPTEC (Jansen *et al.*, 2014) but not in MDCK cell line (Goh *et al.*, 2002; Quan *et al.*, 2012; Gartzke & Fricker, 2014) could explain this difference in results. Although the high lipophilicity of digoxin and the active uptake of digoxin are contradictory, in most studies, a transwell transport assay is used to study vectorial P-gp-mediated digoxin transport. Because in this assay, the permeability of the test compound can be determined in the polarized cells with localized uptake and the efflux transporters (Pauli-Magnus *et al.*, 2001a; Rathore *et al.*, 2003; Balimane *et al.*, 2004; Balimane & Chong, 2005). It seems that, lipophilicity of digoxin is not the only factor playing a role in cellular uptake and there might be other physicochemical properties involved (Pastan *et al.*, 1988; Polli *et al.*, 2001; Yamazaki *et al.*, 2001). Another explanation for lack of digoxin transport by P-gp in MDCK cell might be the presence of Gly185Val mutation in the originally cloned P-gp in our MDCK cell line. It is known that this mutation affects the substrate affinity (Safa *et al.*, 1990; Watanabe *et al.*, 2000) and therefore might affect the digoxin transport.

Since MDCK cell lines originated from canine kidney, they contain endogenous transporters that may lead to false-positive or false negative results for detection of a substrate (Kuteykin-Teplyakov *et al.*, 2010). Based on FDA suggestions (Food and Drug Administration (FDA), 2006; Gartzke & Fricker, 2014), we have used both MDCK and MDCK-P-gp cell lines to prevent this effect (chapter 5). Moreover, to specify the transporters that were involved in the DLCs transport in the MDCK and ciPTEC cellular assays, the inhibitors elacridar and Ko143 were used. Although BCRP expression is lacking in MDCK (Quan *et al.*, 2012; Gartzke & Fricker, 2014), it is present in ciPTEC (Jansen *et al.*, 2014), and the inhibition of digitoxin and proscillaridin A transport in the presence of Ko143 could be an indication for the transport of these DLCs by BCRP.

Future perspective

In this thesis, we have applied different *in vitro* models to study the transport and the interaction of DLCs by drug influx and efflux transporters. We showed that the physicochemical properties of DLCs such as lipophilicity could influence the results of *in vitro* assays and therefore should be considered in the selection of the *in vitro* assays to study their transport.

We argued that the membrane vesicular transport assay is an appropriate tool for screening of transport inhibitors, but neither this assay nor the cellular accumulation assay alone would be sufficient to detect all substrates of the ATP-dependent efflux transporters studied. Since different *in vitro* models are available, these different models should be used and the presence of efflux and influx transporters should be discussed.

To improve the narrow therapeutic index of DLCs, it would be interesting to focus on DLCs that are not transported by the influx and efflux transporters, because these compounds would not be victim of DDIs and resulting toxicity. Based on our studies, gitoxigenin has the least interaction with the influx and efflux transporters. Gitoxigenin, however, is a less potent inhibitor of Na,K-ATPase compared to digitoxigenin, digitoxin, digoxigenin, digoxin and gitoxin (De Pover & Godfraind, 1982; Cornelius *et al.*, 2013). The ideal candidate should contain these structural criteria to interact with Na,K-ATPase: a *cis* conformation of the C/D rings, an unsaturated lactone ring, a β attachment of the lactone ring to C17, a β attachment of the hydroxyl group at positions C3 and C14, and a β attachment of C3 with the sugar moiety (Chen & Henderson, 1954; Brown *et al.*, 1962; Thomas *et al.*, 1974; Sevillano *et al.*, 2002). Additionally in the gitoxigenin structure, the presence of a hydroxyl group at C16 position facilitates the formation of a hydrogen bond between C14 and C16 leading to the formation of a D-ring.

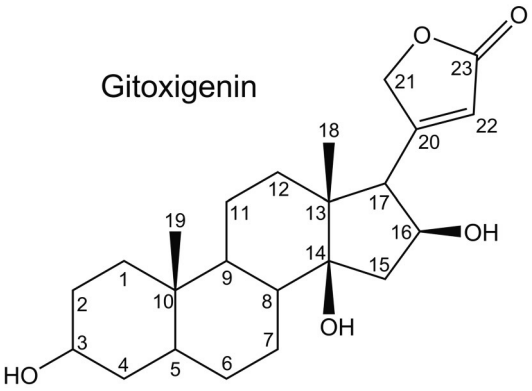


Figure 4. Gitoxigenin. Chemical structure of gitoxigenin has been presented.

This unusual ring alters the position of the lactone ring in the binding pocket of Na,K-ATPase and therefore, decreases the affinity of gitoxigenin for its receptor but still conserves the inhibitory effect on Na,K-ATPase (Griffin *et al.*, 1986; Hashimoto *et al.*, 1986). In addition, the *16*β-formate and *16*β-acetate derivatives of gitoxigenin have a higher affinity for Na,K-ATPase compared to their parent compound (Griffin *et al.*, 1986; Hashimoto *et al.*, 1986). Like digoxin and digitoxin, gitoxigenin has been extracted from *Digitalis lanata* (Caspi & Lewis, 1967; Go & Bhandary, 1989) and it was recently shown to be a component of the essential oil and methanol extracts of sweet basil *Ocimum basilicum* L. (Lamiaceae) and an Indian seed oil called sal (*Shorea robusta*) that are applied in food industry (Dhara *et al.*, 2010; Hossain *et al.*, 2010).

Information about application of gitoxigenin and its derivatives in therapy and relevant DDI is lacking. Based on Lipinski's rule of five (Lipinski *et al.*, 2001), the molecular weight of 390.5, calculated logP of 2.25, existence of 3 and 5 hydrogen bond donors and acceptors, respectively and the lack of interaction with influx and efflux transporters characterize gitoxigenin and its derivatives as good candidates for further investigation to replace digoxin and digitoxin, and reduce DLCs adverse effect and toxicity.

Chapter 8

English Summary

Nederlandse Samenvatting

(Persian Summary) خلاصه فارسی

List of Abbreviations and Glossary

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Acknowledgment

English Summary

Introduction

Digitalis-like compounds (DLCs), also named cardiac glycosides, are produced as protective agents against herbivores or predators by plants and animals, such as fox glove (*Digitalis purpurea*) and *Bufo Bufo* toad, respectively. Moreover, they are known as the ancient treatment of heart failure and atrial fibrillation and currently digoxin and digitoxin are still applied in the clinic. Apart from their different physicochemical and pharmacological properties, DLCs share the inhibitory effect on Na,K-ATPase. Digoxin and digitoxin are characterized by their narrow therapeutic index and adverse effects, which rank them among the top ten drugs leading to hospitalization. Particularly, drug-drug interactions (DDI) at the transporter level play a key role in high plasma concentrations of these DLCs leading to toxicity.

Transport proteins, located in blood-brain barrier, liver, intestine and kidney, prevent accumulation of drugs and potentially toxic compounds in the body. The ATP-binding cassette (ABC) protein superfamily consists of efflux transporters that translocate drugs from inside of the cells to the blood, bile, intestinal lumen and urine. Influx transporters, like organic anion-transporting polypeptides (OATPs) in the liver, translocate drugs from the blood side of the cells to the inside thereby facilitating excretion via efflux transporters. DDI may occur at the level of efflux and influx transporters thereby influencing drug disposition, which is one of the key determinants of drugs safety and efficacy.

In this thesis, we studied the interaction of efflux and influx transporters with a group of structurally related DLCs. Moreover, we investigated the chemical features of DLCs that are important for the interaction with transporters. In addition, we have explored the role of different amino acids in the transport activity of the major drug efflux transporter P-glycoprotein/ ABCB1 (P-gp). We have also identified DLCs that are poor substrates or inhibitors of efflux and influx transporters and introduced DLCs that had the least interactions with transporters, as they might be safer therapeutic alternatives to digoxin and digitoxin.

The role of efflux transporters in DLC excretion

The distribution and function of ABC transporters play a key role in the absorption, distribution, metabolism, and excretion of drugs. Induction or inhibition of these transporters is the underlying mechanism of many drug-drug interactions and adverse drug effects.

In **chapter 2**, we showed that most DLCs interact with BCRP/ABCG2, BSEP/ABCB11, MRP1/ABCC1, MRP2/ABCC2, MRP3/ABCC3, and MRP4/ABCC4 in inside-out membrane vesicles overexpressing the respective transporters. BCRP-mediated transport activity was completely inhibited by 1000 μM digitoxin, digoxin, and peruvoside. Digitoxin was the only potent BSEP inhibitor among the tested DLCs, whereas peruvoside and strophanthidol inhibited BSEP by 50%. Strophanthidol was the strongest MRP1 and MRP3 inhibitor and digitoxigenin was the most potent MRP2 and MRP4 stimulator and inhibitor, respectively.

The typical DLC structure consists of a core steroid ring, which contains a sugar moiety or a hydroxyl group on position C3 and a lactone ring at position C17. In addition, there can be substitutions like a hydroxyl group or a carbonyl group at positions C1, C5, C11, C12, C16, and C19. We showed that chemical substitutions at C1, C3, C11, C12, C16, and C19 positions influence the interaction of DLCs with BCRP, BSEP, and MRPs. Furthermore, the inhibitory effect of DLCs on Na,K-ATPase activity was used to develop a Na,K-ATPase-[^{3}H]ouabain replacement assay to determine their uptake by BCRP, BSEP and MRPs expressing membrane vesicles. Although most DLCs inhibited the activity of the efflux transporters, none of them were transported as a substrate in the vesicle assay, except for convallatoxin by P-gp, which was further confirmed in chapter 4.

In **chapter 3**, we studied the interaction of a series of DLCs with P-gp in a vesicular transport assay. Digitoxin and proscillaridin A were the most potent inhibitors of P-gp and digoxin, peruvoside, and strophanthidol inhibited transport activity by more than 50%. We found that a δ-lactone ring and a sugar moiety at the 3β position of the steroid body are favorable for inhibition of P-gp. In addition, although hydroxyl groups at positions 5β and 19 increased the inhibitory effect of DLCs, inhibition is decreased by a hydroxyl at positions 1β, 11α, 12β, and 16β. To determine the key amino acids of P-gp for interaction with DLCs, ten of them were mutated to alanine. The mutations I306A, F343A, F728A, T945A, and L975A abolished P-gp-mediated transport of the model substrate N-methyl quinidine (NMQ). The affinities of digoxin, proscillaridin A, peruvoside, and cymarin for mutants F336A and I340A were decreased 2- to 4-fold compared to wild type, whereas those of digitoxin and strophanthidol were unaltered. Moreover, the presence of a hydroxyl group at position 12β (digoxin vs. digitoxin) seems to reduce the apparent affinity when the side chain of Phe336 and Phe942 is absent.

In **chapter 4**, we investigated the transport of fourteen DLCs by P-gp in the vesicular transport assay using liquid chromatography-mass spectrometry (LC-MS) for quantification. We identified convallatoxin, Lily of the Valley toxin, as a P-gp substrate. In rat, convallatoxin co-administration with elacridar resulted in increased concentrations in brain and kidney cortex, which confirmed P-gp-mediated convallatoxin transport

in vivo. To determine interacting P-gp residues with convallatoxin, the effect of nine alanine mutations on NMQ transport was compared. We found that Phe343 appeared to be more important for transport of NMQ than convallatoxin, whereas Val982 plays an important role in convallatoxin transport.

In **chapter 5**, a cellular accumulation assay was developed using Madin-Darby canine kidney (MDCK) cells and conditionally immortalized proximal tubular epithelial cells (ciPTECs) of human origin, to study the role of the efflux transporters as gatekeepers to protect cells from toxins. We found that digitoxin, digoxigenin, strophanthidin, and proscillaridin A were substrates of P-gp in both the ciPTEC and MDCK cellular accumulation assays, but not in the vesicular transport assay. In **chapter 4**, we identified convallatoxin as a P-gp substrate; however, it was not transported by P-gp in the cellular accumulation assay. The function of an efflux transporter in the cellular accumulation assay depends on the passive entrance of lipophilic compounds, whereas the vesicular transport assay is more appropriate for hydrophilic compounds that do not cross the vesicular membrane by passive diffusion. Therefore, lipophilic DLCs such as digitoxin, digoxigenin, strophanthidin, and proscillaridin A could not be identified as P-gp substrates in the vesicular assay, whereas the less lipophilic convallatoxin was not detected as P-gp substrate in the cellular accumulation assays. Here, we showed that the transport of substrates with a clogP of - 0.3 and higher could be assessed in a cellular assay, whereas the vesicular assay is suitable for substrates with a lower clogP.

The role of influx transporters in DLC excretion

In **chapter 6**, the interaction of DLCs with the SLC transporters, NTCP/SLC10A1, OATP1B1/SLCO1B1, and OATP1B3/SLCO1B3 were studied in a cellular uptake assay. NTCP transport activity was inhibited most potently by proscillaridin A, however, digitoxin, and digitoxigenin were the most potent inhibitors of OATP1B1 and OAPTP1B3, respectively. Moreover, the presence of a sugar moiety at the C3 position enhanced the inhibition of OATP1B1 transport, whereas it decreased the inhibition of NTCP and OATP1B3 transport. Although the hydroxyl group at position 12 increased the inhibition of NTCP, it reduced the inhibitory potency against OATP1B1 and OATP1B3 activity. Here, we showed for the first time that convallatoxin, ouabain, dihydroouabain, and ouabagenin are substrates of OATP1B3, which implies that this liver uptake transporter could be a potential site of DDI.

Conclusion and future perspectives

In this thesis, we used *in vitro* and *in vivo* models to study the interaction of structurally-related DLCs with clinically relevant drug efflux and influx transporters. In **chapter 7**, we discussed that DLCs, which have the least interactions with these transporters, could be selected as candidate drugs that are less prone to DDIs. Based on our studies, gitoxigenin fulfills these criteria and it could be considered as a replacement for digoxin and digitoxin, which have a high incidence of DDIs and toxicity. In addition, we concluded that the application of one type of *in vitro* transport assay is not sufficient to screen for substrates, because the functionality of vesicular and cellular transport assays depend on the lipophilicity of the substrate.

Nederlandse Samenvatting

Introductie

Digitalisachtige verbindingen (Digitalis-like compounds, DLCs) of hartglycosiden worden geproduceerd door planten en dieren, zoals vingerhoedskruid (Digitalis purpurea) en bruine pad (Bufo Bufo), als beschermende stoffen tegen herbivoren en roofdieren. Digoxine en digitoxine zijn bekend als klassieke geneesmiddelen bij de behandeling van hartfalen en atrium fibrilleren en worden nog steeds voorgeschreven. DLCs verschillen in farmacokinetische kenmerken maar delen alle hetzelfde werkingsmechanisme, namelijk remming het enzym Na,K-ATPase. Ze worden gekenmerkt door een smalle therapeutische index waardoor ze behoren tot de top tien van medicijnen die leiden tot ziekenhuisopname. Geneesmiddeleninteracties (drug-drug interactions, DDIs) op het niveau van de transporteiwitten spelen een belangrijke rol bij het ontstaan van hoge plasmaconcentraties van DLCs welke kunnen leiden tot levensbedreigende toxiciteit.

Transporteiwitten komen onder meer voor in de bloed-hersenbarrière, lever, darmen en nieren en hebben als functie om accumulatie van geneesmiddelen en potentieel schadelijke stoffen in het lichaam te voorkomen. Transportereiwitten die tot de ATP-binding-cassette (ABC) superfamilie behoren, zijn zogenaamde effluxtransporters die stoffen vanuit de cellen actief naar bloed, gal, darmlumen en urine transporteren. Daarnaast zijn er ook influxtransporters zoals de 'organic anion transporting polypeptides' (OATPs) in de lever, die zorgen voor opname van stoffen uit het bloed in de cel, zodat ze vervolgens via effluxtransporters uitgescheiden kunnen worden. Zowel op het niveau van efflux- als influxtransporters kunnen DDIs plaatsvinden die de eliminatie en daarmee de veiligheid van een geneesmiddel nadelig beïnvloeden.

In dit proefschrift hebben we de moleculaire interactie van efflux- en influxtransporters met een groep DLCs met verschillende chemische structuren bestudeerd en hebben we onderzocht hoe de verschillende chemische zijgroepen van de DLCs hierop invloed hebben. Verder hebben we gekeken naar de rol van een aantal aminozuren in de substraatbindingplaats van de effluxtransporter P-glycoproteïne/ABCB1 (P-gp). We hebben ook bepaald welke DLCs substraten van de efflux- en influxtransporters zijn en welke juist de minste interacties met deze transporters geven, omdat dit mogelijk goede kandidaten zijn om interactiegevoelige geneesmidelen zoals digoxine en digitoxine te vervangen.

De rol van effluxtransporters in de eliminatie van DLCs

De lokalisatie en functie van ABC-transporters spelen een belangrijke rol bij de absorptie, distributie, biotransformatie en eliminatie van geneesmiddelen. Inductie of remming van de activiteit van deze transporters is een belangrijk mechanisme voor DDIs en daaruit voortkomende bijwerkingen.

In **hoofdstuk 2** hebben we laten zien dat de meeste DLCs een interactie aangaan met BCRP/ABCG2, BSEP/ABCB11, MRP1/ABCC1, MRP2/ABCC2, MRP3/ABCC3 en MRP4/ABCC4. Deze interacties werden bepaald in membraanblaasjes (vesikels) geïsoleerd van cellen waarin deze transporteiwitten tot overexpressie zijn gebracht. De transportactiviteit van BCRP werd volledig geremd door 1000 μM digitoxine, digoxine en peruvoside. Digitoxine was de sterkste remmer van BSEP, terwijl peruvoside en strophanthidol 50% remming gaven. Strophantidol was de sterkste remmer van MRP1 en MRP3, terwijl digitoxigenine de sterkste remmer en stimulator van respectievelijk MRP2 en MRP4 is.

De typiche structuur van een DLC bestaat uit een steroïdring die de kern vormt, een suiker- of hydroxylgroep op positie C3 en een lactonring op positie C17. Op de posities C1, C5, C11, C12, C16 en C19 kunnen substituenten als een hydroxyl- of een carbonylgroep voorkomen. Wij hebben laten zien dat substituenten op posities C1, C3, C11, C12, C16 en C19 de interacties met BCRP, BSEP en de MRPs beïnvloeden. Het principe van Na,K-ATPase remming door DLCs werd gebruikt om een Na,K-ATPase-[^{3}H]ouabain-inhibitie-test te ontwikkelen. Met deze test kunnen we de opname van remmende DLCs in BCRP-, BSEP- en MRPs-vesikels bepalen. Hoewel de meeste DLCs een interactie met een efflux transporter aangaan, werd geen van deze DLCs als substraat opgenomen in de vesikels, behalve convallatoxine door P-gp dat verder is uitgezocht in hoofdstuk 4.

In **hoofdstuk 3** hebben we de interactie van een groep DLCs met P-gp in membraanvesikels onderzocht. Digitoxine en proscillaridine-A waren de sterkste remmers van P-gp en digoxine, maar ook peruvoside en strophanthidol, remden P-gp meer dan 50%. We toonden aan dat een δ-lactonring en een suikergroep op de 3β-positie van de DLCs gunstig is voor remming van P-gp. De aanwezigheid van hydroxylgroepen op posities 5β en 19 verhogen het remmende effect, terwijl hydroxylgroepen op posities 1β, 11α, 12β, en 16β de remming verminderen. Tien aminozuren van P-gp werden gemuteerd naar alanine om hun rol in de interactie met DLCs te bepalen. De mutaties I306A, F343A, F728A, T945A en L975A blokkeerden het transport van het modelsubstraat N-methyl quinidine (NMQ) door P-gp. De affiniteit voor digoxine, proscillaridin A, peruvoside en cymarin van de mutanten F336A en I340A was 2- tot 4-keer afgenomen in vergelijking met de controle (wild-type), terwijl de affiniteiten van digitoxine en strophanthidol niet waren veranderd. Bovendien zorgde een hydroxylgroep op positie

12β (digoxine vs digitoxine) voor een vermindering van de affiniteit bij afwezigheid van de zijketen van Phe336 en Phe942.

In **hoofdstuk 4** is de opname van veertien DLCs door P-gp in membraanvesikels met behulp van vloeistofchromatografie-massaspectrometrie (LC-MS) gemeten. We identificeerden convallatoxine, het lelietje-van-dalen-toxine, als een P-gp substraat. In ratten verhoogde elecridar de convallatoxine-concentratie in de hersenen en de niercortex, hetgeen bevestigt dat deze DLC *in vivo* ook door P-gp wordt getransporteerd. Om de aminozuren te bepalen die een rol in de convallatoxin-P-gp-interactie spelen, werd van negen alaninemutanten het convallatoxine-transport vergeleken met dat van N-methyl quinidine (NMQ). We vonden een belangrijke rol voor Phe343 in het NMQ-transport, terwijl Val982 juist een belangrijke rol had bij het transport van convallatoxine.

In **hoofdstuk 5** is een meetmethode ontwikkeld om met Madin-Darby canine kidney II (MDCK) cellen en conditionally immortalized proximal tubular epithelial cells (ciPTECs) van humane afkomst de rol van de effluxtransporters die de cellen beschermen tegen toxines te onderzoeken. In ciPTEC- en MDCK-cellen waren digitoxine, digoxigenine, strophanthidin en proscillaridin A substraten van P-gp, terwijl dit uit de vesikelexperimenten niet bleek. In hoofdstuk 4 hebben we laten zien dat convallatoxin een P-gp-substraat was, maar dit kwam niet naar voren in de cellulaire assays. De functie van een effluxtransporter in de cellulaire assays is afhankelijk van de opname van lipofiele substraten in de cellen, terwijl de vesikels juist geschikt zijn voor hydrofiele DLCs omdat deze de membranen niet passief kunnen passeren. Daarom konden lipofiele DLCs zoals digitoxine, digoxigenine, strophanthidine en proscillaridine-A niet worden geïdentificeerd als P-gp-substraten in de vesikulaire assay, terwijl het minder lipofiele convallatoxine niet als P-gp-substraat in cellen werd gedetecteerd. We concluderen dat transport van substraten met een clogP van -0,3 en hoger in cellen kan worden bepaald, terwijl bij een lagere clopP de vesikulaire assay moet worden gebruikt.

De rol van influxtransporters in de eliminatie van DLCs

In **hoofdstuk 6** hebben we onderzocht of DLCs een interactie hebben met de SLC-transporters, NTCP/SLC10A1, OATP1B1/SLCO1B1 en OATP1B3/SLCO1B3. Proscillaridine-A was de sterkste remmer van NTCP-transportactiviteit, terwijl digitoxine en digitoxigenine de sterkste remmers van respectievelijk OATP1B1 en OAPTP1B3 waren. Een suikergroep op de C3-positie verbeterde de remming van OATP1B1, terwijl de remming van NTCP en OATP1B3 hierdoor juist minder werd. Bovendien versterkte een hydroxylgroep op positie 12 de remming van NTCP, maar verminderde het de remming van OATP1B1 en OATP1B3. We toonden hier voor het eerst aan dat convallatoxine, ouabaïne, dihydroouabaïne en ouabagenine substraten zijn van OATP1B3, waardoor DDIs met deze middelen op het niveau van deze influxtransporter kunnen optreden.

Conclusie en toekomstperspectieven

In dit proefschrift hebben we *in vitro*- en *in vivo*-modellen gebruikt om de interactie van verschillende DLCs met efflux- en influxtransporters te onderzoeken. In **hoofdstuk 7** beschrijven we hoe een DLC met de minste transporterinteracties geselecteerd kan worden als kandidaat geneesmiddel met minimale DDIs. Op basis van ons onderzoek had gitoxigenine de minste interacties met efflux- en influxtransporters en is het daarom een mogelijk alternatief voor digoxine en digitoxine, welke een hoge incidentie van DDIs en toxiciteit hebben. Daarnaast hebben we geconcludeerd dat het gebruik van alleen cellen of membraanvesikels niet voldoende is om de substraten van een transporter te onderzoeken. Het resultaat van de assay is namelijk afhankelijk van de lipofiliciteit van de substraten.

خلاصه فارسی

مقدمه

مواد شبه دیجیتالیس (دی‌ال‌سی ها) یا گلیکوزیدهای قلبی به عنوان یک وسیله دفاعی توسط گیاهانی چون گل انگشتانه و جانورانی چون وزغ بوفوبوفو تولید می‌شوند. این مواد به عنوان درمان سنتی نارسایی قلبی و فیبریلاسیون دهلیزی شناخته شده‌اند. در حال حاضر، نمونه‌هایی از این مواد مانند دیگوکسین و دیجی‌توکسین جهت اهداف درمانی مورد استفاده قرار می‌گیرند. مواد شبه دیجیتالیس با وجود ویژگی‌های متفاوت شیمی-فیزیکی و فارماکولوژیکی، همگی جزو مهارکننده‌های پمپ سدیم-پتاسیم محسوب می‌شوند. تفاوت ناچیز بین دوز درمانی و دوز کشنده‌ی این مواد و اثرات جانبی آنها سبب شده تا این مواد در بین ده دارویی که منجر به بستری شدن در بیمارستان می‌گردند، قرار گیرند. تداخلات دارویی در سطح ناقلین دارویی (ترانسپورترها) یکی از مهم‌ترین عوامل بالا رفتن غلظت دی‌ال‌سی ها در پلاسما و نهایتا مسمومیت و مرگ حاصل از این داروها محسوب می‌شود.

ناقلین دارویی در سد خونی-مغزی، کبد، روده و کلیه قرار گرفته و از تجمع داروها و مواد سمی در بدن جلوگیری می‌کنند. خانواده پروتئین‌های (ATP-binding cassette, ABC) جزو ناقلین دارویی افلاکس به شمار می‌آیند که داروها را از داخل سلول‌ها به خون، صفرا، لومن روده و ادرار منتقل می کنند. ناقلین دارویی اینفلاکس مانند پلی پپتیدهای انتقال‌دهنده آنیون‌های ارگانیک (OATP) داروها را از محیط خارج سلولی به داخل سلول‌ها جهت دفع آن‌ها توسط کبد و کلیه انتقال می‌دهند. تداخلات دارویی می‌توانند در هر دو سطح ناقلین ایفلاکس و اینفلاکس رخ دهند و دفع داروها را که یکی از عوامل کلیدی در ایمنی و اثربخشی داروها است، تحت تاثیر قرار دهند.

در این پایان‌نامه، به مطالعه برهمکنش مواد شبه دیجیتالیس و ناقلین دارویی افلاکس و اینفلاکس پرداختیم. همچنین، اثر ساختار شیمیایی دی‌ال‌سی ها بر برهمکنش بین ناقلین دارویی و این مواد موردبررسی قرار گرفته و دی‌ال‌سی هایی نیز به عنوان سوبسترای ناقلین افلاکس و اینفلاکس شناسایی شدند. علاوه براین، در کنار شناسایی آمینواسید هایی که در عملکرد پی-گلیکوپروتئین (P-gp) نقش داشتند، تلاش شده است تا مواد شبه دیجیتالی با حداقل برهمکنش با ناقلین دارویی، به عنوان جایگزین مناسب برای دیگوکسین و دیجی‌توکسین ارائه شوند.

نقش ناقلین افلاکس در دفع مواد شبه دیجیتالیس از بدن

نحوه توزیع و عملکرد ناقلین افلاکس در بدن نقش کلیدی در جذب، توزیع، متابولیسم و دفع داروها دارد. القا یا مهار ناقلین دارویی، مکانیسم اصلی تداخلات دارویی و اثرات جانبی حاصل از آنها است. ما در **فصل دو**، با استفاده از وزیکول‌های غشایی که پروتئین‌های ناقل را بیان می‌کردند، نشان دادیم که بیشتر دی ال سی ها با ناقلین دارویی BCRP/ABCG2، BSEP/ABCB11، MRP1/ABCC1، MRP3/ABCC3، MRP2/ABCC2 وMRP4/ABCC4 برهمکنش دارند. ۱۰۰۰ میکرومولار دیگوکسین، دیجی‌توکسین و پرواوساید عملکرد پروتئین BCRP را کاملا مهار می نمودند.

دیجی‌توکسین تنها مهارکننده پروتئین BSEP بود درحالی که پرواوساید و استروفانتیدول عملکرد BSEP را تا ۵۰ درصد مهار نمودند. همچنین استروفانتیدول، MRP1 و MRP3 را با قدرت بیشتری در مقایسه با MRP4 مهار می‌نمود. اگرچه هیچ مهارکننده‌ای برای پروتئین MRP2 درمیان داروهای مورد مطالعه یافت نشد، دیجی‌توکسی ژنین عملکرد MRP2 را ۳,۶ برابر کنترل، تحریک می‌نمود.

ساختار شیمیایی دی ال سی ها از یک حلقه مرکزی استروئیدی تشکیل شده که دارای یک گروه قندی یا یک گروه هیدروکسیل روی کربن شماره ۳ و یک حلقه لاکتونی روی کربن ۱۷ می باشد. کربن های شماره ۱، ۵، ۱۱، ۱۲، ۱۶ و ۱۹ نیز می توانند دارای گروه های هیدروکسیل و در بعضی مورد دارای گروه کربونیل باشند. ما نشان دادیم که گروه‌های شیمیایی موجود روی کربن‌های شماره ۱ و ۱۱ به صورت همزمان، و کربن‌های شماره ۳، ۱۲، ۱۶ و ۱۹، برهم کنش دی‌ال‌سی ها با BCRP، BSEP و MRPها را تحت تاثیر قرار می‌دهند. همچنین اثر مهاری دی‌ال‌سی ها بر پمپ سدیم-پتاسیم مورد استفاده قرار گرفت تا جذب دی‌ال‌سی ها توسط وزیکول‌های غشایی بیان‌کننده BCRP، BSEP و MRPها بررسی شود. اگرچه اکثر دی‌ال‌سی ها با ناقلین افلاکس ذکرشده در بالا برهم‌کنش داشتند، اما هیچ‌کدام به جز کونوالاتوکسین که توسط P-gp (فصل چهار) منتقل می‌گردید، به عنوان سوبسترا شناسایی نشدند.

در **فصل سه**، ما به مطالعه گروهی از دی‌ال‌سی ها در وزیکول‌های غشایی بیان‌کننده پی-گلیکوپروتئین پرداختیم. دیجی‌توکسین و پراسیلاریدین به عنوان قوی‌ترین مهارکننده‌های پی-گلیکوپروتئین شناسایی شدند و دیگوکسین، پرواوساید و استروفانتیدول عملکرد این پروتئین را تا ۵۰ درصد مهار نمودند. ما نشان دادیم که حلقه لاکتونی-دلتا و گروه قندی موجود روی کربن ۳-بتا، برهم کنش دی‌ال‌سی ها با پی-گلیکوپروتئین را تسهیل می‌کنند. همچنین، اگرچه گروه‌های هیدروکسیل موجود روی کربن های ۵ و ۱۹، اثر مهاری دی‌ال‌سی ها را افزایش می‌دهند، در صورت قرارگیری روی کربن های ۱، ۱۱، ۱۲ و ۱۶ می‌توانند اثر مهاری دی‌ال‌سی ها را کاهش دهند.

جهت تعیین آمینواسیدهای کلیدی در برهم کنش دی‌ال‌سی ها با P-gp، ده آمینواسید P-gp به آلانین جهش داده شدند. جهش ایزولوسین ۳۰۶، فنیل‌آلانین ۳۴۳، فنیل‌آلانین ۷۲۸، ترئونین ۹۴۵ و لوسین ۹۷۵ به آلانین، سبب از دست رفتن عملکرد P-gp در انتقال سوبسترای مدل آن، ان-متیل کینیدین (NMQ) شدند. تمایل دیگوکسین، پراسیلاریدین، پرواوساید و سی‌مارین به پروتئین‌های دارای جهش فنیل‌آلانین ۳۳۶ و ایزولوسین ۳۴۰ به آلانین، دو تا چهار برابر نسبت به حالت وحشی پروتئین کاهش یافت. درحالی که تمایل دیجی‌توکسین و استروفانتیدول برای این پروتئین‌های جهش یافته فاقد تغییر بود. همچنین در صورت مقایسه‌ی دیگوکسین با دیجی‌توکسین، به نظر می‌رسد که وجود گروه هیدروکسیل در موقعیت ۱۲-بتا سبب کاهش تمایل دی‌ال‌سی ها به پروتئین‌های دارای جهش فنیل‌آلانین ۳۳۶ و فنیل‌آلانین ۹۴۲ به آلانین می‌شود.

در **فصل چهار** با اندازه‌گیری غلظت دی‌ال‌سی ها در وزیکول‌های غشایی با استفاده از تکنیک کروماتوگرافی مایع-طیف‌سنجی جرمی (LC-MS)، به بررسی انتقال چهارده دی‌ال‌سی توسط P-gp پرداختیم. کونوالاتوکسین، سم موجود در گل موگه، به عنوان سوبسترای P-gp در وزیکول‌های غشایی شناسایی شد. تزریق کونوالاتوکسین به همراه الاکریدار (مهارکننده P-gp) به موش‌های صحرایی، سبب افزایش غلظت کونوالاتوکسین در مغز و کورتکس کلیه گردید که

نشان‌دهنده نقش P-gp در انتقال این ماده بود. برای تعیین آمینواسیدهای P-gp که در انتقال کونوالاتوکسین نقش دارند، نه آمینواسید P-gp به آلانین جهش داده شدند و عملکرد پروتئین‌های جهش‌یافته در انتقال کونوالاتوکسین با عملکرد آن‌ها در انتقال NMQ مقایسه گردید. ما دریافتیم که فنیل‌آلانین ۳۴۳ در انتقال NMQ نسبت به انتقال کونوالاتوکسین توسط P-gp، نقش مهم‌تری دارد درحالی که والین ۹۸۲، آمینواسید ضروری جهت انتقال کونوالاتوکسین به شمار می‌رود.

در **فصل پنج**، از تست تجمع سلولی جهت بررسی نقش ناقلین افلاکس به عنوان محافظت‌کنندگان سلولی دربرابر تجمع دی‌ال‌سی ها در سلول‌های کلیوی سگ (MDCK) و انسان استفاده شد. ما نشان دادیم که دیجی‌توکسین، دیگوکسی‌ژنین، استروفانتیدین و پراسیلاریدین، سوبسترای P-gp در سلول‌های کلیوی سگ و انسان هستند، درحالی که این مواد به عنوان سوبسترای P-gp در وزیکول‌های غشایی شناسایی نشدند. کونوالاتوکسین که به عنوان سوبسترای P-gp در فصل چهار شناسایی شده بود، در تست تجمع سلولی به عنوان سوبسترا شناسایی نشد. عملکرد یک ناقل ایفلاکس در سلول‌ها نیازمند ورود مواد چربی‌دوست به داخل سلول به صورت غیرفعال می‌باشد درحالی که وزیکول‌های غشایی بیشتر برای مواد آبدوستی که نمی‌توانند از غشا به صورت غیرفعال عبور کنند، مناسب است. بنابراین دی‌ال‌سی های چربی‌دوستی مانند دیجی‌توکسین، دیگوکسی‌ژنین، استروفانتیدین و پراسیلاریدین به عنوان سوبسترای P-gp در وزیکول‌های غشایی قابل شناسایی نیستند درحالی که مواد آبدوستی چون کونوالاتوکسین نیز به عنوان سوبسترای P-gp در تست سلولی عمل نمی‌کنند. همچنین ما نشان دادیم که سوبستراهایی با ضریب تفکیک اکتانول به آب ۰٫۳- و یا بالاتر در تست‌های سلولی قابل مطالعه و شناسایی هستند.

نقش ناقلین اینفلاکس در دفع سلول‌ها از بدن

در **فصل شش**، به مطالعه برهمکنش ناقلینی چون NTCP/SLC10A1، OATP1B1/SLCO1B1 و OATP1B3/SLCO1B3 با دی‌ال‌سی ها در سلول پرداختیم. در این مطالعه، پراسیلاریدین به عنوان قوی‌ترین مهارکننده NTCP و دیجی‌توکسین و دیجی‌توکسی ژنین به ترتیب به عنوان قوی‌ترین مهارکننده‌های OATP1B1 و OATP1B3 شناسایی شدند. علاوه براین وجود گروه قندی روی کربن ۳ دی‌ال‌سی ها سبب تسهیل مهار OATP1B1 توسط این مواد می‌شد، درحالی که سبب کاهش مهار NTCP و OATP1B3 می‌گردید. اگرچه وجود گروه هیدروکسیل روی کربن ۱۲ دی‌ال‌سی ها، مهار NTCP را افزایش می‌داد، مهار عملکرد OATP1B1 و OATP1B3 توسط این گروه شیمیایی کاهش می‌یافت. در اینجا، برای اولین بار کونوالاتوکسین، وابائین، دی‌هیدرو وابائین و واباژنین به عنوان سوبسترای OATP1B3 شناسایی شدند و پروتئین هایی که مسؤول جذب داروها در کبد هستند نیز، عنوان سطحی برای بروز تداخلات دارویی معرفی گردیدند.

نتیجه‌گیری و چشم‌انداز آینده

در این پایان‌نامه از مدل‌های *in vitro* و *in vivo*، جهت مطالعه برهم‌کنش گروهی از مواد شبه دیجیتالیس با ناقلین افلاکس و اینفلاکس استفاده گردید. در **فصل هفت**، دی‌ال‌سی هایی که حداقل برهم‌کنش با ناقلین دارویی داشتند، به عنوان جایگزین مناسب با کمترین تداخلات دارویی مطرح گردیدند. براساس مطالعه ما، جی‌توکسی ژنین که کمترین برهم کنش را با ناقلین ایفلاکس و اینفلاکس داشت، می‌تواند جایگزین مناسبی برای دیگوکسین و دیجی‌توکسین که دارای شانس بالای تداخلات دارویی و مسمومیت هستند، باشد. شایان ذکر است که از آنجا که عملکرد تست‌های سلولی و وزیکولی به چربی‌دوستی سوبستراها وابسته است، به کارگیری یک نوع تست *in vitro* برای مطالعه عملکرد ناقلین دارویی و شناسایی سوبستراهای آنها کافی نیست.

List of abbreviations and glossary

A	Adenosine, a nucleobase found in DNA and RNA
A	Alanine, a hydrophobic amino acid
ABCB1	ATP-binding cassette B1/ gene name for P-gp
Ala	Alanine, a hydrophobic amino acid
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
AP1	Activating protein 1
Asn	Asparagine, a polar amino acid
Asp	Aspartic acid, a charged amino acid
ATP	Adenosine triphosphate
AV	Atrioventricular
BCRP	Breast cancer resistance protein
BSA	Bovine serum albumin
BSEP	Bile salt export pump
C	Cytosine, a nucleobase found in DNA and RNA
Caco-2	Human epithelial colorectal adenocarcinoma
CHO	Chinese hamster ovary
ciPTEC	Conditionally immortalized proximal tubule epithelial cell
DDI	Drug-drug interaction
DIG	Digitalis Investigators Group
DLC	Digitalis-like compounds
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
E_1S	Estrone 3-sulfate
$E_2$17βG	β-estradiol 17-β-D-glucuronide
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal-regulated kinase
eYFP	Enhanced yellow fluorescent protein
F	Phenylalanine, a hydrophobic amino acid
FCS	Fetal calf serum
FDA	Food and drug administration
G	Guanine, a nucleobase found in DNA and RNA
Gln	Glutamine, a polar amino acid
HBSS	Hanks balanced salt solution
HEK	Human embryonic kidney cell line
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, a buffering agent
HESI	Heated electrospray ionization

I	Isoleucine, a hydrophobic amino acid
IC_{50}	The concentration at which 50% of maximum rate of the enzyme is inhibited
IGEPAL CA-630	Octylphenoxypolyethoxyethanol, a nonionic, non-denaturing detergent
Ile	Isoleucine, a hydrophobic amino acid
IP3	Inositol 1, 4, 5-triphosphate
JNK	Jun NH_2-terminal kinase
K_m	Michaelis-Menten constant, the substrate concentration at which an enzyme reaction rate reaches at 50% of its maximum
L	Leucine, a hydrophobic amino acid
LC-MS	Liquid chromatography-mass spectrometry
Leu	Leucine, a hydrophobic amino acid
LLC-PK1	Epithelial-like pig kidney cell
LogP	Octanol: water partition coefficient
γ-lactone	γ-butyrolactone, four-carbon lactone ring
δ-lactone	δ-valerolactone, five-carbon lactone ring
MAP	Mitogen-activated protein
MDCK	Madin-Darby canine kidney
MDR	Multiple drug resistance
MRP	Multidrug resistance-associated protein
Mus musculus	House mouse
NBD	Nucleotide-binding domain
NF	Nuclear factor
NMQ	N-methyl-quinidine
NTCP	Na^+-dependent taurocholate co-transporting polypeptide
NSAIDs	Nonsteroidal anti-inflammatory drugs
OATP	Organic anion transporting polypeptide
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
P-gp	P-glycoprotein, multidrug resistance protein 1
Phe	Phenylalanine, a hydrophobic amino acid
Pro	Proline, a non-essential amino acid
PROVED	Prospective Randomized Study of Ventricular Function and Efficacy of Digoxin
PSA	Polar surface area
QZ59-RRR	cyclic-tris-(R)-valineselenazole
QZ59-SSS	cyclic-tris-(S)-valineselenazole

RADIANCE	Randomized Assessment of Digoxin on Inhibitors of the Angiotensin Converting Enzyme
Rifampicin	Rifampin, A bactericidal antibiotic that inhibits bacterial RNA synthesis
RIPA	Radio-Immunoprecipitation Assay buffer
SA	Sinoatrial
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SF21	The cell line from *Spodoptera frugiperda*, a moth species and agricultural pest of corn
SIM	Single ion monitoring
SLC	Solute carrier transporters
T	Threonine, a polar amino acid
T	Thymine, a nucleobase found in DNA
TCA	Taurocholic acid
Thr	Threonine, a polar amino acid
TM	Transmembrane
TMD	Transmembrane domain
TS	Tris-HEPES
V	Volume
Val	Valine, a nonpolar amino acid
V_{max}	The maximum reaction rate of an enzyme
VSV-G	Vesicular stomatitis virus
W	Weight
Xenopus laevis	African clawed frog

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Curriculum vitae

Elnaz Gozalpour was born on August 6th, 1983, in Tehran, Iran. She achieved her diploma in Natural Sciences from Prof. Hesabi High school in Tehran in 2001. Immediately thereafter, she started her bachelor's study in Cellular and Molecular Biology at Tehran University and graduated in 2005. She pursued her master's education in Human Genetics at the University of Social Welfare and Rehabilitation Sciences in Tehran. During her master's project she focused on the association of cholesterol metabolism genes with the risk of sporadic Alzheimer's disease in an Iranian population. In addition, she was also involved in a similar project in Iranian patients with multiple sclerosis. After her graduation in February 2009, she joined the Department of Pharmacology and Toxicology at Radboud University Medical Center in May 2009 to start the PhD research described in this thesis under the supervision of prof. dr. F.G.M. Russel and dr. J.B. Koenderink. This project was part of a NWO Vidi grant awarded to dr. J.B. Koenderink. In December 2014, she moved to the UK to take up a position as a drug metabolism and pharmacokinetics scientist at AstraZeneca, a multinational pharmaceutical and biologics company.

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- Gozalpour E, Greupink R, Bilos A, van den Heuvel JMW, Russel FGM, Koenderink JB. Convallatoxin: a new P-glycoprotein substrate, European Journal of Pharmacology, 2014 December 5; 744: 18-27.

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از خواهر و برادران همسرم، فاطمه، ابوذر و مهدی که همواره جویای احوال من بوده اند، و از هیچ گونه محبتی دریغ نکردند ممنونم وبرایشان سلامتی و خوشبختی را در کنار همسران گرامی و فرزندان دلبندشان آرزومندم

پدر و مادر نازنینم، جایگاهی که اکنون در آن قرار دارم بر دشت ایثار و مشقت شما که مملو از گل های عشق و محبت است، در پشت کوه حمایت و پشتیبانی تان و در جوار دریای اشک های دلتنگی مان بنا شده است، آن را بیش از همه به شما مدیونم. فرشتگان زمینی ام، برای سختی های که برایم متحمل شدید و برای تنها گذاشتن شما شرمسارم و برای دنیایی که به من هدیه کرده اید، سپاسگزارم و سلامتی و شادمانی همیشگی تان را آرزومندم.

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